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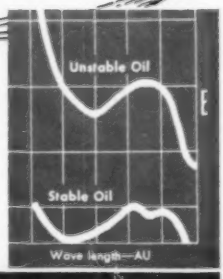

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The Adsorption of Enteroviruses by Activated Attapulgite*

By PASQUALE BARTELL, WALTER PIERZCHALA, and HOWARD TINT

An *in vitro* study was made of the adsorptive capacity of Claysorb for several representative human enteroviruses: poliovirus, types 1, 2, and 3; ECHO-9, and Coxsackie B-3. Within wide limits of virus concentration, temperature, pH, and duration of exposure, no specificity was evident in attaining a saturation adsorption level with a given clay concentration. Claysorb was superior to kaolin in comparison adsorption trials with these agents. These findings suggest a potential use for Claysorb in localized intestinal infections and other syndromes associated with enteroviruses.

THE ADSORPTIVE properties of hydrous aluminum silicate complexes has encouraged the use of natural clays in preparations for treating enteric disturbances of toxic and infectious origin (1). Barr (2) has reviewed the potential for this purpose of activated attapulgite, a hydrated magnesium-aluminum silicate, first described by Lapparent (3). Barr and Arnista (4, 5, 6) have described comparative studies with several clays in alkaloidal, bacterial, and exotoxin (diphtheria) adsorption studies, and have demonstrated a persistent superiority of attapulgite over halloysite and kaolin, although the last is frequently employed as an ingredient in intestinal adsorbent preparations. The recent observation by Neter and Gorzynski (7) that activated attapulgite can also effectively adsorb lipopolysaccharide endotoxins of enteric bacteria has considerably extended the range of potential therapeutic application of this clay. On the other hand, little information is available on the capacity of these clays for other enteropathogenic agents, namely viruses. It appeared desirable to test preparations of adsorptive clays on typical viruses[1] frequently isolated from the human alimentary tract. To this end, the ability of Claysorb[2] to adsorb representative agents was investigated. Studies were carried out *in vitro* of the variables of virus inoculum, pH, time, and temperature upon adsorption; and some comparisons between the adsorptive properties of this clay and kaolin were made.

* Received September 3, 1959 from the Product Development Division, Wyeth Laboratories, Radnor, Pa.

[1] Enteroviruses, a group name prepared for intestinal viruses including poliomyelitis, Coxsackie, and ECHO groups of viruses (8).

[2] Claysorb, trademark of Wyeth, a refined attapulgite with low volatile matter, principally water.

EXPERIMENTAL

Materials.—The clays employed in these studies were obtained from regular commercial sources. Claysorb and kaolin[3] were sterilized dry as received, by autoclaving at 15 pounds, 121°, forty-five minutes. A sterile stock suspension of each clay was prepared as 10% w/v suspension in Hank's balanced salt solution (9) and adjusted to pH of approximately 7.4 with 7.5% sodium bicarbonate solution.

The human enteric viruses selected for study were the following: polioviruses, type I (Brunhilde), type II (YSK), and type III (Leon); ECHO, type 9;[4] Coxsackie, type B-3.[4]

The serially-propagated HeLa cell (10) was used throughout this study as substrate for measuring viral infectivity. The management of this cell line has been adequately described in the literature (11, 12) and only a brief description will be given here. The cells were propagated as monolayers on glass in a growth medium consisting of 20% human adult serum in Eagles' basal medium (13) containing 0.1% dextrose, 100 u./ml. potassium penicillin G, and 50 mcg./ml. dihydrostreptomycin sulfate. The final pH was adjusted to approximately 7.4 with 7.5% sodium bicarbonate solution.

[3] Kaolin, English clays, Lovering Pochin Ltd., St. Austell, England.

[4] These viruses, adapted to growth in the HeLa cell line, were kindly supplied by the South Jersey Medical Research Foundation.

Assay Procedure.—Virus suspensions were titrated in the HeLa tissue culture as follows: test tubes containing healthy cell monolayers were washed three times with Hank's balanced salt solution and then replenished with 0.9 ml. of a maintenance medium containing 10% horse serum according to the formula of Scherer (14). Groups of four test-tube cultures were each inoculated with 0.1 ml. of serial tenfold dilutions of the virus suspensions to be titered. The tubes were then incubated in a stationary position at 37° for seven days with a change of maintenance medium after the third or fourth day. The tubes were examined on the seventh day and the final virus titer was calculated according to the method of Reed and Muench (15). Each titer is expressed as the number of infectious doses infecting 50% of the tissue cultures ($TCID_{50}$) per ml. of original virus suspension.

Methods and Results.—The adsorptive properties of the clays were tested generally as follows: 0.5-ml. aliquots of virus suspension were added individually to 4.5 ml. of stock 10% clay suspension, and to an equal volume of Hank's balanced salt solution as control. The infected clay suspension was thoroughly mixed by shaking, and both experimental and control mixtures were incubated at 37°, in a water bath for forty-five minutes. The clay preparation was then clarified by centrifugation at 1,000 r. p. m. for ten minutes; and the supernatant fluid and the control were simultaneously titrated for virus infectivity in the HeLa-cell system. The difference in titratable activity between the treated fluid and the control then reflected the amount of virus adsorbed by the clay treatment.

The adsorptive capacity of Claysorb for the several enteroviruses is summarized in Fig. 1. It may be seen that at least three logs of infectivity of poliovirus, types 1 and 2, were adsorbed, representing 99.9% of the virus content. Poliovirus type 3, ECHO-9, and Coxsackie B-3 were also adsorbed, but to a lesser degree.

The effect of varying the size of the virus inoculum was determined next. Serial dilutions of poliovirus, type 1, were mixed under standard treatment with the Claysorb stock solution, and residual activity in the respective supernatants again determined. As shown in Fig. 2, a maxium of approximately three logs of virus infectivity was again adsorbed, and this level remained fairly constant for the entire dilution series. It will be noted that with a level of infectivity not exceeding 1,000 infectious doses per ml., the amount of Claysorb in the test procedure was capable of withdrawing all measureable infectivity in the mixture.

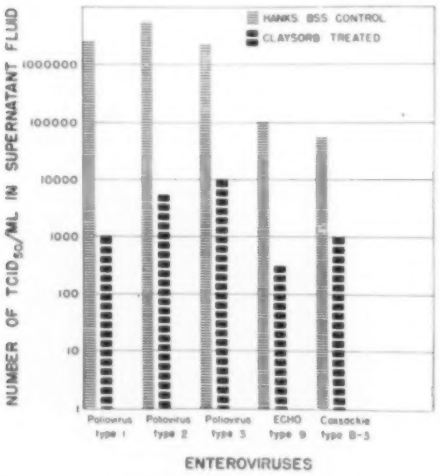

Fig. 1.—The adsorption of enteroviruses by Claysorb (100 mg./ml.).

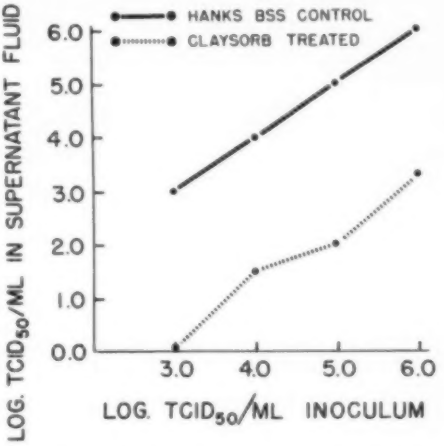

Fig. 2.—The effect of varying virus inoculum on the adsorption of poliovirus, type 1, by Claysorb (100 mg./ml.).

Since intestinal adsorbents must function in the gastrointestinal tract where a wide range of pH prevails, it appeared advisable to test the adsorptive property of Claysorb in a system with comparably variable pH. Suspensions of Claysorb (100 mg./ml.) were prepared in buffered saline at pH levels of 3, 5, 7, and 9 with an acetate-Veronal buffer (16). Poliovirus, type 1, was then added to these several suspensions and the residual infectivity of each supernatant was determined. It will be noted in Fig. 3 that the adsorptive capacity of the clay was fairly constant over the wide pH range tested.

The relationship of time of exposure to adsorption was studied in the standard mixture again with poliovirus, type 1. Separate suspensions were centrifuged at 1,000 r. p. m. immediately after mixing and at several time intervals thereafter, fifteen, thirty, and forty-five minutes. These results are summarized in Fig. 4 and indicate that the adsorption of this virus by Claysorb is immediate and that there is no further adsorption when the exposure is increased fifteen, thirty, and forty-five minutes.

Variation in the temperature of the exposure at fixed levels of 4, 25, and 37°, contributed no significant changes in the quantities of virus adsorbed (Fig. 5).

In several experiments, the comparative adsorptive properties of Claysorb and kaolin were determined in this system and the results of four such comparisons are shown in Fig. 6. With poliovirus, type 1, as the test virus, the Claysorb preparation

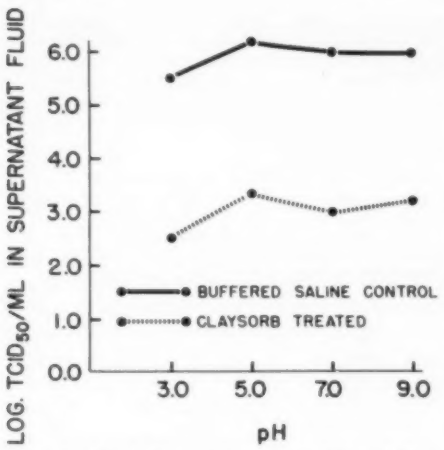

Fig. 3.—The influence of pH on the ability of Claysorb (100 mg./ml.) to adsorb poliovirus, type 1.

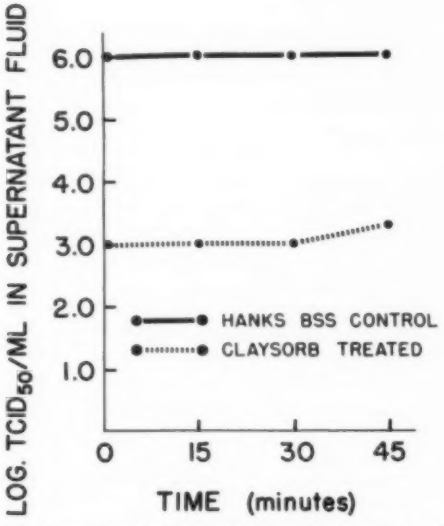

Fig. 4.—The relationship of time to the adsorption of poliovirus, type 1, by Claysorb (100 mg./ml.).

appeared to be five to tenfold more effective in withdrawing virus than that of kaolin at equivalent clay concentrations. This is again evident in Fig. 7 which compares the adsorptive capacity of varying concentrations of Claysorb and kaolin for poliovirus, type 1.

DISCUSSION

It is evident from these results that Claysorb is capable of adsorbing significant quantities of representative human enteroviruses, including all three poliovirus types and typical ECHO and Coxsackie agents. Upon exposure of high concentrations of poliovirus, type 1, to the clay, adsorption of

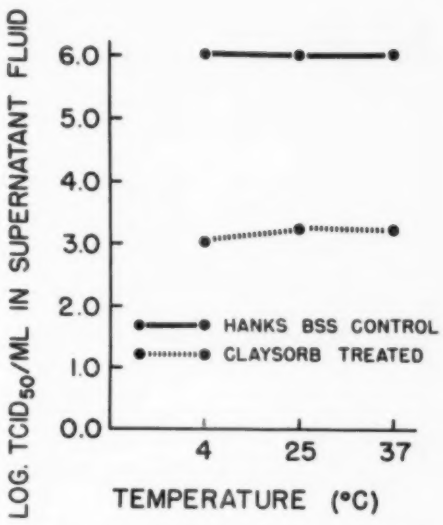

Fig. 5.—The influence of temperature on the ability of Claysorb (100 mg./ml.) to adsorb poliovirus, type 1.

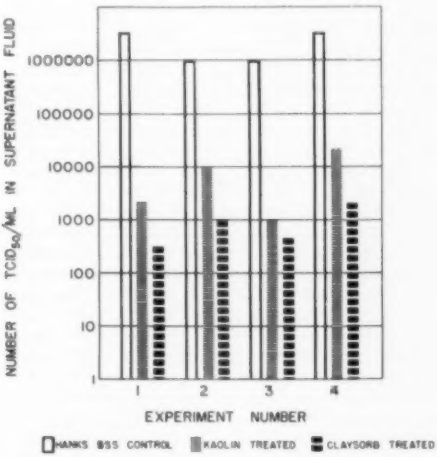

Fig. 6.—The comparative abilities of Claysorb (100 mg./ml.) and kaolin (100 mg./ml.) to adsorb poliovirus, type 1.

up to three logs of virus infectivity is attained, representing 99.9% of the initial infectivity levels. Conversely, virus fluids containing not more than 1,000 infective doses of virus can be treated in the same fashion with Claysorb to yield supernatant fluids substantially free of measureable infectivity. The adsorptive capacity of the clay is operative within limits of pH normally found within the gastrointestinal tract and is apparently independent of temperature and time of exposure within the limits of study. In comparison tests, Claysorb had approximately five to ten times the adsorptive capacity for virus as kaolin under equivalent conditions.

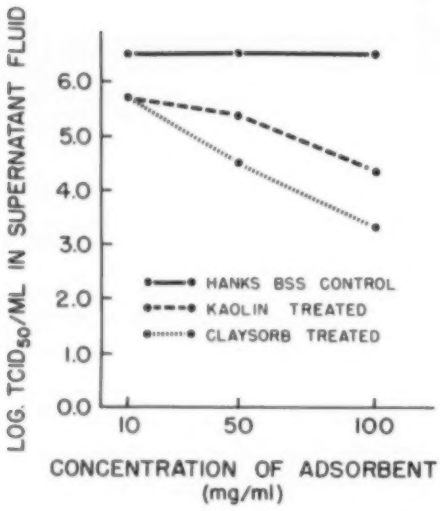

Fig. 7—The effect of varying concentrations of Claysorb and kaolin to adsorb poliovirus, type 1.

The effects of Claysorb on enterovirus adsorption are interesting from a potential clinical point of view, but difficult to assess in prospect. The prevalence of these viruses in the human alimentary tract and the frequency of isolations from stool specimens of patients during illness suggest a strong causal association between these agents and localized intestinal infectivity; for example, in the case of some diarrheas, evidence is being accumulated which strongly suggests that a cause-and-effect relationship may exist with certain enteroviruses (17). On the other hand, for other enterovirus-associated syndromes, many variable factors at play in the gastrointestinal tract, as well as a general lack of knowledge on viral pathogenesis, make proof of this localization difficult to establish. There is no question that poliovirus can be discharged from infected host by alimentary elimination to spread to other individuals by way of ingestion; poliovirus, Coxsackie, and ECHO viruses can frequently be isolated from fecal specimens, and the virus of infectious hepatitis has been experimentally passed to human volunteers through the oral administration of fecal filtrates. The possibility of binding these viruses with preparations of Claysorb points to a potential clinical value of such compositions in limiting viral spread from the infected source, and possibly lessening the infectivity level locally in the secondary host. The realization of these potentials, however, can only be established through appropriate clinical study.

SUMMARY

1. Claysorb at a concentration of 100 mg./ml. effectively adsorbed significant quantities of the infectivity of representative enteroviruses tested: polioviruses, type 1 (Brunhilde), type 2 (YSK), type 3 (Leon); ECHO, type 9, Coxsackie, type B-3.

2. With poliovirus, type 1, as indicator system the adsorptive activity of Claysorb was relatively constant at pH levels of 3, 5, 7, and 9.

3. The adsorptive activity occurred immediately and was not increased with extended exposure. The amount of virus adsorbed remained unchanged with temperature variation: 4, 25, and 37°.

4. Claysorb showed five to ten times greater adsorptive capacity than kaolin, when tested at equivalent clay concentration levels of 5 and 10% for poliovirus, type 1.

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Osmotic Pressure and Tolerance of Injectable Solutions*

By I. SETNIKAR and MARIA ROSA PATERLINI

By determining the degree of irritation caused in the derma by solutions of substances in common pharmaceutical use, it can be shown that these solutions may be divided into two categories: those which exert an osmotic pressure (because they do not pass through the cell membrane) and those which exert no osmotic pressure (because they do pass through the cell membrane). Injectable solutions prepared with substances of the second group must be rendered isotonic by the addition of the same quantity of a product not freely diffusible through the cell membrane which would be necessary to render distilled water isotonic. Furthermore, it has been demonstrated that slightly hypertonic solutions are better tolerated than isotonic solutions and, hence, when desiring to prepare a well tolerated injectable solution of a substance whose osmotic pressure is not known and cannot be determined by a biological method, 0.9 per cent of NaCl may be added.

ON THE BASIS of experiments performed on red cells, it has been demonstrated in a previous paper (1) that it is erroneous and sometimes dangerous to confuse the osmotic pressure of injectable solutions with their osmotic concentration, as determined by measuring the depression of the freezing point or the vapor tension. Many substances pass freely through the membrane of the red cell, thus exerting no osmotic pressure, and so cause hemolysis even when at a concentration iso-osmotic with blood.

It was of interest to check whether these substances diffuse freely also through the membranes of other cells that come into contact with injected liquids, i. e., with the cells of subcutaneous and muscle tissue.

Besides this, it is of great practical interest to know the range of osmotic pressure both above and below the isotonic level still well tolerated by the tissues, because it is not always possible for the pharmacist to prepare injectable solutions in isotonic concentration.

These two problems are the subject of the present report.

METHODS

The osmotic effect upon the tissues was tested by preparing slices of tissue of not more than 1 mm. in thickness and immersing them in solutions of NaCl at different concentrations. The weight differences in the slices of tissue before contact with the solutions in question and after two hours immersion, expressed as a percentage of the initial weight, was an index of the imbibing or disimbibing effect of the solution examined.

The local tolerance was determined by the method of Hoppe, Alexander, and Miller (2), as modified by Koelzer and Wehr (3), i. e., by injecting into the abdominal derma of rats 0.1 cc. of the solution under examination and, immediately after, endovenously, 1 cc./Kg. of a 1% solution of trypan blue dissolved in physiological saline. After three and after twenty-four hours the local irritation set up by the solution was examined and classified as follows: 0, no effect; 1, slight blue coloration; 2, blue coloration with slight inflammation; 3, intense blue coloration with marked inflammation; 4, blue ring around an ischemic center or small necroses; 5, necroses with small eschars.

Sterile NaCl solutions at different concentrations and in doses of 0.1 cc. were injected also into the volar derma of the forearm of human subjects. The pain felt by these subjects was classified as follows: 0, none; 1, slight but definitely perceptible pain; 2 severe pain; 3, very severe and almost unbearable pain.

RESULTS

Imbibing Effect.—Subcutaneous connective tissue slices taken from the thorax of rats, muscle slices taken from the rectus abdominis mucle of rats, and slices of liver and thymus tissue of rats, after two hours contact with NaCl solutions at various concentrations, presented the weight changes given in Table I. The concentration of the solution of NaCl had no effect upon the imbibition by the connective tissue, whose weight in two hours increased steadily by 40–60%, irrespective of the concentration of NaCl. On muscle tissue and, still more, on that of the thymus, the high concentrations of NaCl hindered imbibition. Finally, as regards liver tissue, high concentrations of NaCl produced disimbibition.

TABLE I.—PERCENTAGE WEIGHT CHANGES IN TISSUE SLICES IMMERSED IN SOLUTIONS OF NaCl AT DIFFERENT OSMOTIC CONCENTRATIONS

Tissue	No. of Expts.	Concentrations of NaCl, % 0.5	0.9	1.8	2.7
Connective	4	45	39	51	58
Muscle	4	40	29	17	16
Thymus	4	25	13	11	3
Liver	4	17	15	−0.7	−4

These results can probably be explained by taking account of the presence of the fundamental intercellular substance, which is very avid for water and is not separated from the perfusing solution by any

* Received July 20, 1959, from the Research Dept. of Recordati Laboratorio Farmacologico S.p.A., Milano, Italy.

semipermeable membrane. Therefore, this substance takes up water and masks, from the point of view of weight balance, what is happening in the cells. The same results were obtained when immersing slices of tissue in concentrated solutions of dextran or serum albumin, which certainly attract water from the cells. Table I shows further that the more the fundamental substance of the tissues preponderates the more independent of the NaCl concentration is tissue imbibition. In fact, fundamental tissue substance is present in increasing proportions in the following order: liver, thymus, muscle tissue, subcutaneous connective tissue.

In this experiment a possible source of error might originate by tissue substances which dissolve in the saline solutions altering their osmotic pressure. This mechanism, however, cannot influence the osmotic pressure of the isotonic saline solution and explain the imbibition observed when the tissues were immersed in the 0.9% NaCl solution. The described test is, in any case, of little practical value for those very tissues, the connective and the muscular, which frequently come into direct contact with injected solutions.

Local Tolerance.—The effects of the osmotic pressure of a solution were therefore investigated indirectly by testing the irritation of the tissues caused by solutions at different osmotic concentrations, and the following results were obtained.

Substances Which Are Nondiffusible Through the Cell Membrane.—Intradermal injections of solutions at different concentrations of NaCl and dextrose give rise to an average degree of irritation (average between the degree of irritation at the third hour and at the twenty-fourth hour) given in Fig. 1. The effect of the osmotic concentration upon the tolerance of the solutions was evident but the surprising point was that the concentrations best tolerated were not isotonic but those with an osmotic pressure about twice the isotonic one.

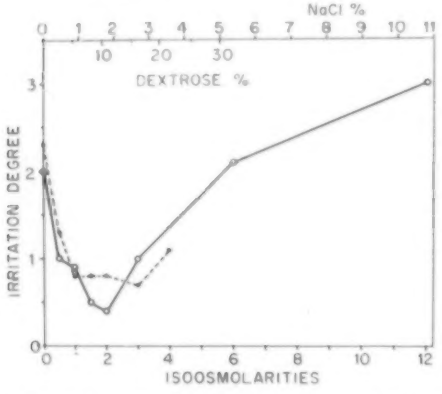

Fig. 1.—Degree of irritation caused by solutions of NaCl and dextrose at different concentrations. ——— NaCl, – – – – dextrose.

Osmotic Concentration and Pain.—This finding was, we felt, of such importance as to deserve confirmation and so the pain effect of solutions of NaCl at different concentrations was tested on human volunteers. The results given in Fig. 2 demonstrate that the concentration which gives rise to least pain is in

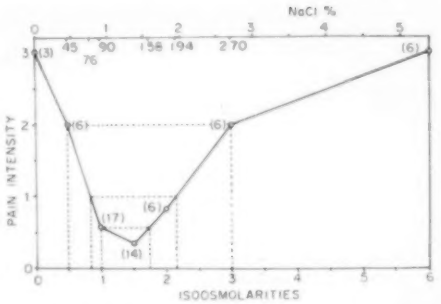

Fig. 2.—Severity of pain caused by intradermal injection of 0.1 cc. of NaCl solutions at different concentrations. The number of experiments, on which the average given in the figures is based, is in brackets.

fact the 1.35% solution of NaCl (1.5 × isotonic). The isotonic concentration of NaCl, i. e., the 0.9% solution, was also very well tolerated but it gives rise to a sensation of pain equal to that caused by a solution 1.75 times the isotonic concentration, i. e., 1.58%. Finally, as regards pain, the extreme concentrations still tolerated had a lower limit of 0.5 × isotonic and an upper limit of 3 × isotonic. Between these limits no reaction of the tissues on the site of the injection could be detected.

Substances Which Are Diffusible Through the Cell Membranes.—The irritant effect of iso-osmotic solutions of succinic dinitryl (2.25%) to which NaCl had been added in graduated concentrations was very illustrative (Table II).

TABLE II.—IRRITANT EFFECT OF AN ISO-OSMOTIC SOLUTION OF SUCCINIC DINITRYL IN THE ABSENCE AND IN THE PRESENCE OF NaCl AT VARIOUS CONCENTRATIONS[a]

Concentration		Degree of Irritation		
Succinic Dinitryl, Gm. %	NaCl Gm. %	After 3 hr.	After 24 hr.	Av.
0	0	2.2 ± 0.1	2.4 ± 0.3	2.3
0	1.35	0.1 ± 0.0	0.0 ± 0.0	0.1
2.25	0	2.3 ± 0.2	2.4 ± 0.4	2.4
2.25	0.45	0.6 ± 0.1	0.4 ± 0.0	0.5
2.25	0.9	0.2 ± 0.1	0.0 ± 0.0	0.1
2.25	1.35	0.2 ± 0.1	0.0 ± 0.0	0.1
2.25	1.80	0.2 ± 0.2	0.0 ± 0.0	0.1

[a] Every mean with its standard error is based upon 5 experiments.

The iso-osmotic solution of succinic dinitryl gave rise to an irritation equal to that of the distilled water, but when from 0.45 to 1.8% of NaCl was added to this solution, the irritation decreased a great deal and became almost equal to the very slight irritation caused by a 1.35% solution of NaCl.

Clearly, succinic dinitryl, of itself, exerts no particularly harmful action and the considerable irritation which follows injections of iso-osmotic solutions of the substance, equal to that observed following injections of distilled water, is due to the incapacity of succinic dinitryl to counterbalance the intracellular osmotic pressure. Results in all respects similar can

be observed with iso-osmotic solutions of ethanol (1.4%) or of propylene glycol (2.4%), substances which, like succinic dinitryl, are freely diffusible through the cell membrane. Substantially similar, though less evident owing to the interference of a certain irritant action proper to the products, were the results obtained with iso-osmotic solutions of urea (1.8%) and of procaine hydrochloride (5.05%).

DISCUSSION

Even though subject to a greater degree of methodological error, the results obtained by testing the local irritation of various substances in ordinary pharmaceutical use substantially confirm those obtained by the hematocrit method (1), *viz.* that many of these substances exert no osmotic pressure because they pass freely through the cell membrane. Thus, when parenteral solutions containing substances freely diffusible through the cell membrane are to be rendered isotonic, the presence of these substances in solution is to be ignored and the same quantity of the isotonizing agent, e. g., NaCl, has to be added as one would add to distilled water.

Very interesting from the practical point of view were the experiments which demonstrate that hypertonic solutions are better tolerated than isotonic ones. Thus, when it is not possible to perform an isotonicity test with a biological method, 0.9% of NaCl may be added to the solution. In this case, if the drug belongs to the group of substances which do not exert osmotic pressure, the solution, after the addition of NaCl, becomes isotonic and is well tolerated. If, however, the drug does exert an osmotic pressure, the solution becomes hypertonic and as such is equally well tolerated. This procedure for preparing injectable solutions well tolerated with regard to osmotic pressure is much simpler and more rational than the rules laid down by various previous investigators for adding NaCl in quantities which have to be determined by formulas, nomograms, or graphs of varying degrees of complexity, but which always ignore the real osmotic pressure exerted by the dissolved substances.

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Analytical Methods for Rescinnamine*

By S. R. MISSAN, L. L. CIACCIO, W. H. McMULLEN, H. J. PAZDERA, and T. C. GRENFELL

Four methods of analysis for rescinnamine are described. These methods are shown to be applicable to the analysis of the pure compound mixtures of reserpine and rescinnamine and pharmaceutical preparations.

WHILE a number of alkaloids have been isolated from *Rauwolfia serpentina* and investigated, the only highly potent tranquilizing alkaloid identified until recently was reserpine, which was isolated and studied by Muller, Schlittler, and Bein (1, 2).

In 1954, Cronheim, Stipp, and Brown (3) reported evidence suggesting that reserpine was not the only active alkaloid present in rauwolfia roots. Comparison of reserpine and the alseroxylon fraction indicated that the extract possessed a greater degree of activity than would be accounted for by its reserpine content. Klohs, Draper, and Keller (4, 5) first reported the isolation and identification of rescinnamine from the alseroxylon fraction.

Although a number of papers have appeared reporting analytical methods for reserpine in whole root, root extracts, and pharmaceutical preparations, only a few analytical methods have appeared for the determination of rescinnamine in roots (6, 7) and none have been reported for pharmaceutical preparations. This paper will deal primarily with the methods of identification and analysis of pure rescinnamine and rescinnamine in dosage forms and mixtures of reserpine and rescinnamine.

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EXPERIMENTAL

Ultraviolet Absorbance.—The ultraviolet spectrum of rescinnamine was first reported by Klohs, *et al.* (4), and Carol and his co-workers (6) utilized the ultraviolet absorbance of 3,4,5-trimethoxybenzoic acid and 3,4,5-trimethoxycinnamic acid for the determination of reserpine and rescinnamine in chromatographic fractions after alkaline hydrolysis. With certain precautions, the measurement of the ultraviolet absorbance of rescinnamine is the basis for a quantitative method of analysis.

Since rescinnamine may be hydrolyzed fairly easily, reserpic acid, methyl reserpate, and trimethoxycinnamic acid may be present in dosage forms. These compounds interfere with the quantitative absorbance measurement and must be removed. By measuring the amount of trimethoxycinnamic acid or reserpic acid removed during the process, the amount of decomposition that has occurred may also be approximated. The amount of trimethoxycinnamic acid present is the best indication of decomposition since reserpic acid is not too stable and decomposes

to give products with altered absorbance spectra. Figure 1 shows the ultraviolet spectra of rescinnamine, reserpic acid, and trimethoxycinnamic acid in chloroform. Figure 2 shows the spectra of reserpic acid in 0.01 *N* HCl and in 1% $NaHCO_3$ and trimethoxycinnamic acid in 1% $NaHCO_3$. Figure 3 shows the calibration curve of rescinnamine in chloroform. (Chloroform solutions of rescinnamine are stable provided they are kept in low actinic glassware.)

Assay of Dosage Forms.—Dosage forms such as tablets may be assayed by the following procedure: *Reagents.*—6 *M* aqueous hydrochloric acid; chloroform, A. R.; 1% (w/v) sodium bicarbonate; 2% (w/v) citric acid.

Preparation of Standard Curve.—Prepare solutions of standard rescinnamine in chloroform (use low actinic glassware) containing 0.002 to 0.02 mg./cc. Measure the absorbance at 308 mμ in a 1-cm. cell using chloroform in the reference cell. Plot absorbance *vs.* concentration.

Procedure for Tablets.—Weigh a counted number of not less than twenty tablets and reduce them to a fine powder. Weigh an amount equivalent to 1.25 mg. of rescinnamine into a low actinic 125-cc. separatory funnel containing 20 cc. of chlorofom and extract with 20 cc. of 6 *M* hydrochloric acid solution. Transfer the chloroform solution to a second separatory funnel and wash the hydrochloric acid with 10 cc. of chloroform. Draw off the chloroform layer and combine with the chloroform from the first extract. Wash the combined chloroform extracts with 20 cc. of 2% citric acid and transfer the chloroform extract to a third separatory funnel. Extract with 20 cc. of 1% sodium bicarbonate solution. Filter the chloroform solution through anhydrous sodium sulfate into a low actinic 100-cc. volumetric flask. Wash the bicarbonate solution with 10 cc. of chloroform, combine with the chloroform solution in the volumetric flask, and dilute to the mark with chloroform. Measure the absorbance at 308 mμ in a 1-cm. quartz cell using chloroform in the reference cell.

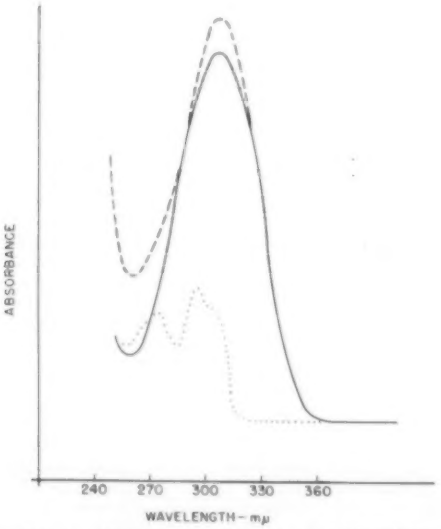

Fig. 1.—Absorbance spectra of 3,4,5-trimethoxycinnamic acid (——), rescinnamine (- - - -), and reserpic acid hydrochloride in chloroform (. . . . .). Determined on a Cary recording spectrophotometer model 11 using 2-cm. cells.

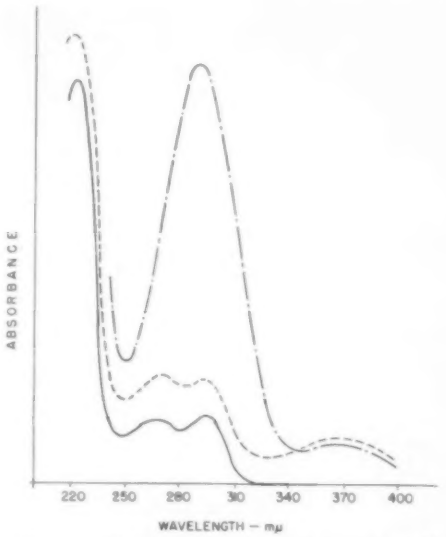

Fig. 2.—Absorbance spectra of reserpic acid hydrochloride (——) in 0.01 *M* hydrochloric acid, 3,4,5-trimethoxycinnamic acid (·····) and reserpic acid hydrochloride (- - - -) in 1% $NaHCO_3$ solution. Determined on a Cary recording spectrophotometer model 11 using 2-cm. cells.

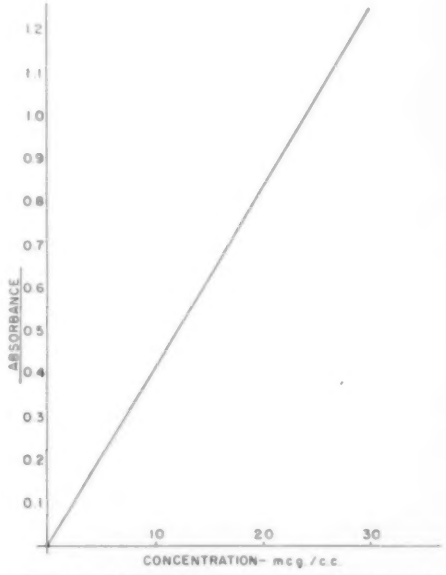

Fig. 3.—Calibration curve of rescinnamine in chloroform. Determined on a model DU Beckman quartz spectrophotometer using 1-cm. cells.

The ultraviolet characteristics of reserpine and rescinnamine are such that rescinnamine can be determined in the presence of fairly large amounts of reserpine by measuring the absorbances at 268 mμ and at 308 mμ.

The amount of rescinnamine and reserpine in a chloroform solution can be calculated from the following formulas:

$$\text{Rescinnamine mcg./cc.} = \frac{(A_2 \times E_1 - A_1 \times E_2) \times 10^4}{(E_1 \times E_4 - E_2 \times E_3)}$$

$$\text{Reserpine mcg./cc.} = \frac{(A_1 \times E_4 - A_2 \times E_3) \times 10^4}{(E_1 \times E_4 - E_2 \times E_3)}$$

where A_1 = absorbance at 268 mμ, A_2 = absorbance at 308 mμ, $E_1 = A^{1\%}_{1\text{ cm.}}$ of standard reserpine at 268 mμ, $E_2 = A^{1\%}_{1\text{ cm.}}$ of standard reserpine at 308 mμ, $E_3 = A^{1\%}_{1\text{ cm.}}$ of standard rescinnamine at 268 mμ, and $E_4 = A^{1\%}_{1\text{ cm.}}$ of standard rescinnamine at 308 mμ.

Figure 4 shows ultraviolet absorbance spectra for mixtures of rescinnamine and reserpine. Table I shows the recoveries of known mixtures of rescinnamine and reserpine.

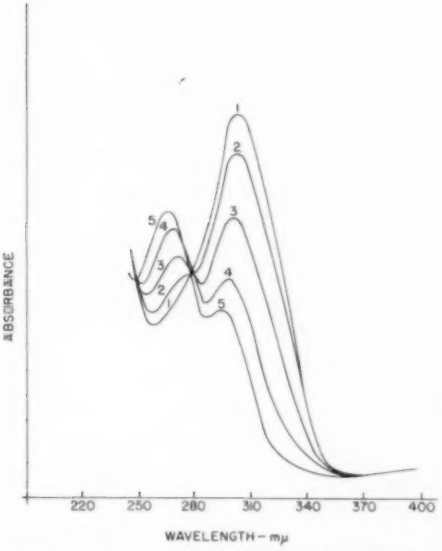

Fig. 4.—Ultraviolet absorbance spectra of mixtures of rescinnamine and reserpine in chloroform. Determined on a Cary recording spectrophotometer model 11 using 2-cm. cells. Curve 1—90% rescinnamine, 10% reserpine; curve 2—75% rescinnamine, 25% reserpine; curve 3—50% rescinnamine, 50% reserpine; curve 4—25% rescinnamine, 75% reserpine; and curve 5—10% rescinnamine, 90% reserpine.

Fluorometric Methods.—Both reserpine and rescinnamine have a natural fluorescence in 3 *M* acetic acid. At an activation wavelength of 310 mμ, rescinnamine fluoresces at 455 mμ whereas reserpine has no fluorescence. The fluorescent intensity is proportional to the rescinnamine concentration. Figure 5 shows the activation and fluorescent spectra of rescinnamine in 3 *M* acetic acid and Fig. 6 shows a typical standard curve. This method is not as sensitive as the nitrite method which is discussed below and the fluorescence is easily swamped out by the fluorescence of the oxidation products from either rescinnamine or reserpine.

TABLE I.—MIXTURES OF RESERPINE AND RESCINNAMINE DETERMINED BY ULTRAVIOLET MEASUREMENT

Sample	Reserpine, % Added	Reserpine, % Found	Rescinnamine, % Added	Rescinnamine, % Found
1	10	10.5	90	90
2	25	24.6	75	75
3	50	50.2	50	49.9
4	75	75	25	24.8
5	90	90	10	9

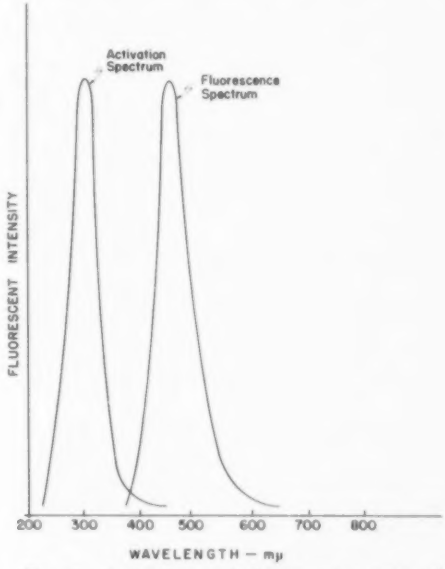

Fig. 5.—Activation and fluorescent spectra of rescinnamine in 3*M* acetic acid. Determined on an Aminco - Bowman recording spectrophotofluorometer.

Rescinnamine can also be determined by the formation of a fluorescent oxidation product. The fluorescent spectrum is identical with that of reserpine so that the method does not differentiate between the two compounds. A fluorometric procedure for reserpine was first reported by Kelly and Poet (8) who used selenious acid as the oxidizing agent. McMullen, *et al.* (9), reported the ultraviolet spectrum and fluorescent spectrum of the reserpine derivative. They used a mixed acid for its production. Szalkowski and Mader (10) reported the formation of the fluorescent product from both reserpine and rescinnamine by the treatment with sodium nitrite and dilute sulfuric acid. They determined only the ultraviolet spectrum and used this as the quantitative method. Banes and his co-workers (11) modified the procedure by removing excess nitrite with sulfamic acid. In a subsequent article, Banes (12) reported that the oxidation product was a 3-dehydroreserpate.

Although this method does not differentiate be-

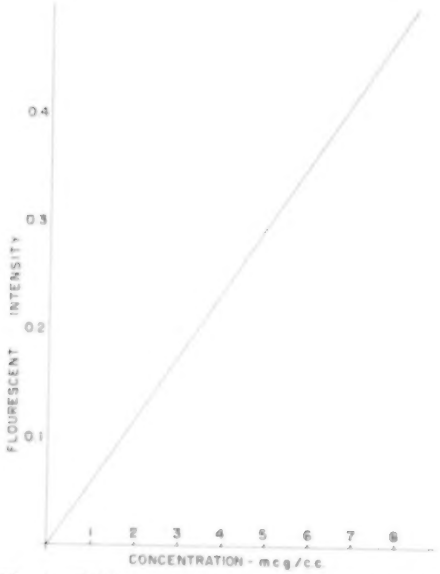

Fig. 6.—Calibration curve for rescinnamine in 3 *M* acetic acid.

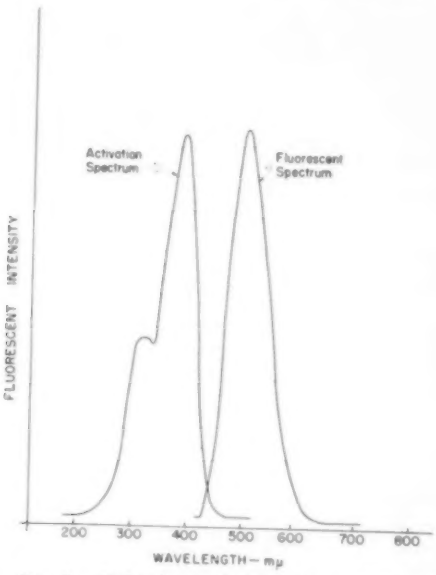

Fig. 7.—Activation and fluorescent spectra of rescinnamine after oxidation with sodium nitrite. Determined on an Aminco-Bowman recording spectrophotofluorometer.

tween rescinnamine and reserpine, the extreme sensitivity of the fluorometric method makes it a desirable assay. With the Aminco-Bowman spectrophotofluorometer, less than 10^{-6} mg./cc. can be detected in contrast with 5×10^{-3} mg./cc. by ultraviolet absorbance.

Reagents.—Unless otherwise indicated, all reagents used are reagent grade. Anhydrous methanol; hydrochloric acid; sodium nitrite, 0.3% in 50% methanol-water, sulfamic acid, 5% aqueous solution.

Standard Rescinnamine Solutions.—Prepare a 5 mcg./cc. solution of rescinnamine in 20% (v/v) chloroform in methanol. Use low actinic glassware throughout.

Procedure.—Pipet 0.5 to 5-cc. aliquots of rescinnamine solution into a 50-cc. volumetric flask. Bring to 5 cc. with 20% (v/v) chloroform solution in methanol. Add 1.0 cc. of sodium nitrite solution and 0.2 cc. hydrochloric acid. Let stand thirty minutes and then add 0.5 cc. of sulfamic acid solution. Dilute to mark with methanol. Measure fluorescence at 500 mμ using an activating wavelength of 385 mμ.

The activation and fluorescent spectra are shown in Fig. 7 and a typical calibration curve is shown in Fig. 8.

Because of the large range of sensitivity and slit combinations of the Aminco-Bowman spectrophotofluorometer, virtually any concentration of rescinnamine can be determined by this method. The fluorescence of the rescinnamine solutions should be determined without the sodium nitrite (use 0.5 cc. H_2O instead) to correct for fluorescent impurities. However, standard rescinnamine prepared in this laboratory had no fluorescence due to impurities and the natural fluorescence does not interfere because of the difference in the activation wavelength and low concentrations used.

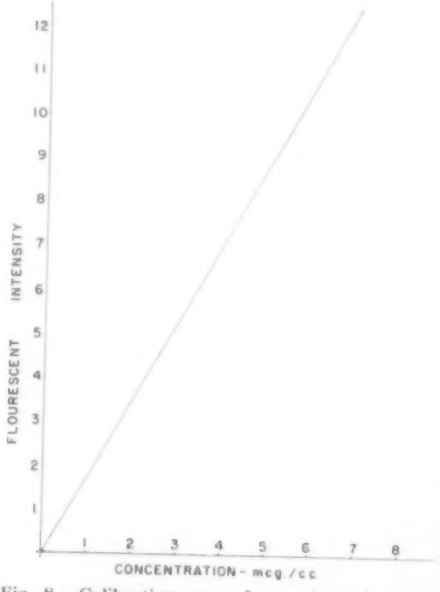

Fig. 8.—Calibration curve for rescinnamine after oxidation with sodium nitrite.

Procedure for Assay of Tablets.—Transfer a 20-cc. aliquot of chloroform solution as prepared under the ultraviolet procedure to a 100-cc. low actinic volumetric flask and dilute to mark with methanol. Transfer 5-cc. aliquots to two 50-cc. low actinic volumetric flasks. To the contents of one flask, add

0.3 cc. of water and 0.2 cc. of hydrochloric acid. To the other, add 1.0 cc. of sodium nitrite solution and 0.2 cc. of hydrochloric acid and let both flasks stand thirty minutes. Add 0.5 cc. of sulfamic acid solution to each and dilute to mark with methanol. Measure the fluorescence of each solution as previously described. Subtract the fluorescent intensity of the blank from the sample. It is advisable to run a standard along with the sample because the intensity of the xenon arc of the spectrophotofluorometer changes with time.

Bromination Method.—The rescinnamine content of rescinnamine-reserpine mixtures can be determined easily by taking advantage of the reactivity of the double bond of trimethoxycinnamic acid. When rescinnamine is brominated, there is a decrease in the ultraviolet absorbance at 315 mμ which is proportional to the concentration of rescinnamine. Because reserpine is not brominated, there is no change in its ultraviolet characteristics.

Figure 9 shows a typical calibration curve obtained by this procedure. The ultraviolet spectra of brominated and unbrominated rescinnamine against a reagent blank are shown in Fig. 10. Figure 11 shows the same data for 3,4,5-trimethoxycinnamic acid. The ultraviolet absorbance spectrum of unbrominated rescinnamine with brominated rescinnamine in the reference cell is shown in Fig. 12.

Reagents.—Bromine in glacial acetic acid, 1% (v/v); 1.2% (v/v) hydrazine hydrate in glacial acetic acid; chloroform A.R.; 5 *M* acetic acid. Use low actinic glassware for all solutions containing rescinnamine.

Preparation of Standard Curve.—Prepare chloro-

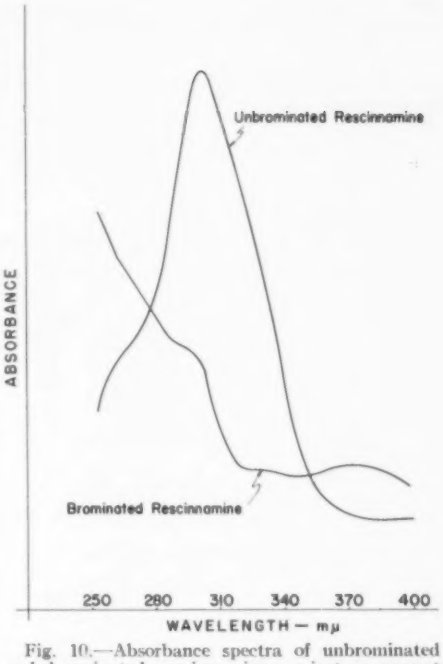

Fig. 10.—Absorbance spectra of unbrominated and brominated rescinnamine against a reagent blank. Determined on a Cary recording spectrophotometer model 11 using 2-cm. cells.

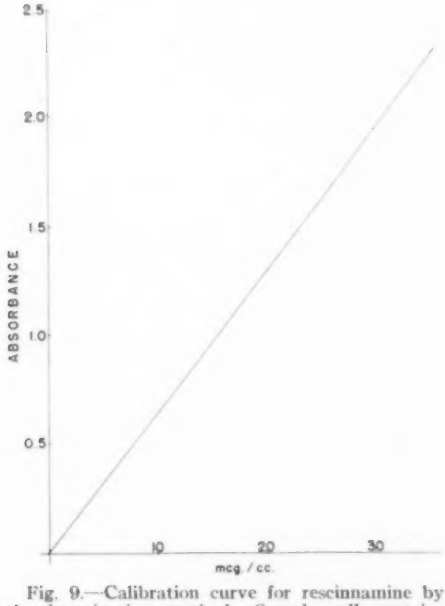

Fig. 9.—Calibration curve for rescinnamine by the bromination method. Sample cell contains unbrominated rescinnamine and reference cell contains brominated rescinnamine. Determined on a Beckman model DU quartz spectrophotometer using 1-cm. cells.

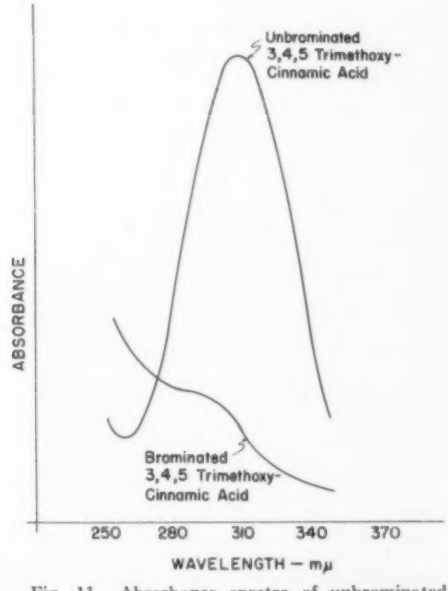

Fig. 11.—Absorbance spectra of unbrominated and brominated 3,4,5-trimethoxycinnamic acid against a reagent blank. Determined on a Cary recording spectrophotometer model 11 using 2-cm. cells.

TABLE II.—PRECISION OF ANALYSIS

Sample	Label Potency	Amount Added	Ultraviolet	Fluorometric		Bromination
				Nitrite	3 *M* Acetic Acid	
			Pure Rescinnamine, %			
1	100	...	100.0	100.0	99.6	99.6
2	100	...	99.8	99.5	100.1	100.0
3	100	...	99.6	99.8	102.0	99.7
4	100	...	101.1	100.0	98.8	99.0
	<Av ($\bar{x}$)>	...	100.1	99.0	100.1	99.6
	<σ>	...	0.67	0.24	1.36	0.42
			Trituration, mg./Gm.			
1	10	...	10.0	10.3	...	10.0
2	10	2	11.9	12.1	...	12.0
3	10	5	15.2	14.8	...	14.8
4	10	7	16.8	16.9	...	17.6
			Tablets, mg./tablet[a]			
1	0.250	...	0.250	0.245	...	0.249
2	0.250	0.025	0.279	0.283	...	0.280
3	0.250	0.050	0.300	0.310	...	0.305
4	0.250	0.0750	0.325	0.325	...	0.323
			Elixir, mg./cc.			
1	0.100	...	0.094	0.102	...	0.083
2	0.100	0.020	0.125	0.114	...	0.104
3	0.100	0.050	0.151	0.144	...	0.133
4	0.100	0.100	0.193	0.201	...	0.182

[a] The tablets were reduced to a fine powder in a mortar and weighed amounts of rescinnamine added to the crushed tablets

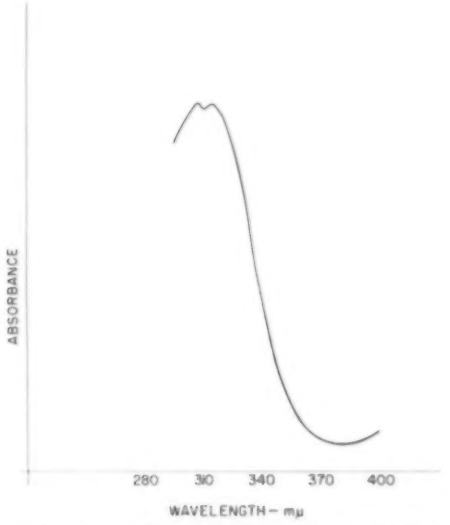

Fig. 12.—Absorbance spectrum of unbrominated rescinnamine with brominated rescinnamine in the reference cell. Determined on a Cary recording spectrophotometer model 11 using 2-cm. cells.

form solutions of standard rescinnamine to contain 20 to 100 mcg./cc. Transfer 5-cc. aliquots of each solution to two 25-cc. volumetric flasks. To one flask, add 1 cc. of bromine solution and let stand fifteen minutes at room temperature. Add 2 cc. of hydrazine solution, mix well, and add 1 cc. of 5 *M* acetic acid to dissolve the precipitate which forms. Then dilute the contents of both flasks to volume with glacial acetic acid. Determine the absorbance at 315 mμ of the unbrominated solution using the brominated solution in the reference cell. Plot the concentration of rescinnamine *vs.* absorbance.

Procedure for Assay of Tablets.—Weigh an amount of finely pulverized tablets equivalent to 0.5 to 2.5 mg. of rescinnamine into a 60-cc. separatory funnel and extract the rescinnamine by the procedure given under the ultraviolet method. However, half of prescribed volumes are used and the final volume of the chloroform solution is 25 cc. Transfer 5-cc. aliquots to two 25-cc. low actinic volumetric flasks and proceed as in the preparation of the standard curve.

The actual determination is made with brominated rescinnamine in the reference cell and not with a reagent blank.

Procedure for Assay of Rauwolfia Roots.—*Reagents.*—Ammonium hydroxide, 28% A.R.; 95% ethanol; peroxide-free diethyl ether; 1 *N* sulfuric acid; chloroform, A.R.; anhydrous sodium sulfate; 2% (w/v) citric acid solution, 1% (w/v) sodium bicarbonate solution.

Procedure.—Accurately weigh a 2-Gm. sample of powdered root (60 mesh or finer) into a 250-cc. Erlenmeyer flask. Add 6 cc. of ethanol, 4 cc. of 28% ammonium hydroxide, and 200 cc. of peroxide-free diethyl ether. Stopper and shake for three hours. Let the mixture settle for ten minutes. Decant 100 cc. into a 250-cc. separatory funnel and extract three times with 20 cc. of 1 *N* sulfuric acid. Combine the acid extracts and filter through a pledget of cotton into a 250-cc. separatory funnel. Extract the sulfuric acid solution three times with 30-cc. portions of chloroform. Combine the chloroform extracts in a 250-cc. separatory funnel and extract once with 20 cc. of 2% citric acid solution and once with 20 cc. of 1% sodium bicarbonate solution. Filter the chloroform extract through anhydrous sodium sulfate. Evaporate the chloroform solution to 1 cc. on a steam bath under a stream of nitrogen. Chromatogram the entire chloroform concentrate by the pro-

cedure of McMullen, *et al.* (9). (This chromatographic procedure will not separate reserpine from rescinnamine.) The amounts of reserpine and rescinnamine can be determined by the two point formula. Rescinnamine alone can be determined by the bromination procedure.

TABLE III.—RESULTS OF ANALYSIS OF VARIOUS DOSAGE FORMS

Sample	Label Potency	Ultraviolet	Fluorometric Nitrite	Bromination
		Trituration, mg./Gm.		
1	10	10.0	10.0	10.1
2	10	9.8	9.9	9.7
3	10	10.1	10.1	10.2
4	10	10.3	10.3	10.0
	<Av. ($\bar{x}$)>	10.1	10.1	10.0
	<σ>	0.21	0.17	0.22
		Tablets, mg./tablet		
11	0.250	0.248	0.248	0.249
12	0.250	0.247	0.250	0.245
13	0.250	0.250	0.249	0.252
14	0.250	0.253	0.252	0.250
	<Av. ($\bar{x}$)>	0.249	0.250	0.249
	<σ>	0.0027	0.0017	0.0029
15	0.500	0.510	0.508	0.510
16	0.500	0.495	0.500	0.497
17	0.500	0.498	0.505	0.500
18	0.500	0.512	0.515	0.510
	Av. ($\bar{x}$)	0.504	0.507	0.504
	σ	0.0085	0.0063	0.0067
		Elixir mg./cc.		
21	0.10	0.096	0.100	0.094
22	0.10	0.098	0.100	0.102
23	0.10	0.098	0.102	0.096
24	0.10	0.102	0.100	0.096
	Av. ($\bar{x}$)	0.098	0.100	0.098
	σ	0.0024	0.0010	0.098

DISCUSSION

Although all four methods are equally valid for pure rescinnamine, we have found that the ultraviolet method substantiated by the bromination method is preferred for bulk rescinnamine. Considerable fluorescence from traces of oxidative impurities interferes with the direct fluorescence of rescinnamine in acetic acid. If the rescinnamine is extremely pure, good results are obtained by this method. The method, therefore, serves as a quick check as to the purity of rescinnamine. For tablets and triturations, the ultraviolet method, the fluorometric method after oxidation with sodium nitrite, and the bromination method are equally valid. However, the method of choice is the fluorometric method after oxidation with sodium nitrite. The bromination method, however, is not valid for elixirs, possibly because of the very small concentration of rescinnamine. For crude rauwolfia preparations, the methods of choice are the ultraviolet method using the two point formula and the bromination method.

Table II shows the precision of the four methods applied to pure rescinnamine, triturations, tablets, and elixirs. Table III shows the results of analysis of various dosage forms and Table IV shows the recoveries of pure rescinnamine mixed with varying amounts of reserpine. Table V shows the recoveries of rescinnamine and reserpine from rauwolfia roots by the two point ultraviolet method and rescinnamine by the bromination method after paper chromatography.

TABLE IV.—RESCINNAMINE DETERMINED IN THE PRESENCE OF RESERPINE BY THE BROMINATION METHOD

Sample	Reserpine, %	Rescinnamine, %	Rescinnamine Found, %
1	4.5	95.5	95.5
2	9.0	91.0	91.0
3	16.5	83.5	83.5
4	22.0	78.0	78.0
5	28.5	71.5	71.4

TABLE V.—RESERPINE AND RESCINNAMINE CONTENT OF RAUWOLFIA ROOTS

	Ultraviolet Method		Bromination Method
Sample	Rescinnamine by Weight, %	Reserpine by Weight, %	Rescinnamine by Weight, %
1	0.081	0.19	0.082
2	0.078	0.21	0.076
3	0.083	0.22	0.083
4	0.080	0.21	0.080
5	0.077	0.19	0.075

SUMMARY

Four methods for the determination of rescinnamine are described. The methods are shown to yield accurate results when applied to pure or crude rescinnamine and various pharmaceutical preparations.

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The Biosynthesis of C^{14}-Labeled Ergot Alkaloids*

By A. G. PAUL†, N. J. SCULLY‡, and A. E. SCHWARTING§

The biosynthesis of C^{14}-labeled ergot alkaloids was achieved by growing rye plants infected with *Claviceps purpurea* in an atmosphere of $C^{14}O_2$. Alkaloids isolated proved to be radioactive. On storage, however, decomposition of the alkaloids was observed. It is suggested that this decomposition may have been caused by the inherent ionizing radiation.

THE INCORPORATION of radioactive isotopes into drugs provides a sensitive method of detecting these materials in quantities which cannot be accurately detected by the usual chemical and biological techniques. Where no practical chemical synthesis is available, biosynthesis must be relied upon for producing these labeled compounds. Examples of medicinal agents which have been uniformly labeled with C^{14} by biosynthesis include digitoxin and nicotine (1), veratrum alkaloids (2), colchicine (3), morphine (4), and reserpine (5).

A successful attempt at biosynthesizing C^{14}-labeled ergot alkaloids was reported by Paul and Schwarting (6) in 1955. The success of this trial experiment warranted an attempt at biosynthesis on a larger scale.

EXPERIMENTAL

Canadian spring rye was grown in two-gallon crocks containing washed sand and fed with modified Hoagland's nutrient solution containing $Ca(NO_3)_2$, 0.005 *M*; KH_2PO_4, 0.0025 *M*; $MgSO_4$, 0.0015 *M*; B, 0.50 p. p. m.; Zn, 0.05 p. p. m.; Mn, 0.25 p. p. m.; Cu, 0.02 p. p. m.; Mo, 0.05 p. p. m.; Fe, 0.50 p. p. m. added as Sequestrene NaFe.

The plants were maintained on a schedule of sixteen hours light and eight hours dark for forty-five days. At the end of this time, twenty crocks of rye were placed in an Argonne-type plant growth chamber (7) of 10,000 L. air capacity.

The crocks were immersed in a bed of sand which was connected to a tank containing 850 L. of the nutrient solution. The nutrient was pumped into the bed and drained daily throughout the course of the experiment.

The temperature was adjusted to 63° F. and the relative humidity to 70%. The chamber, situated in a greenhouse, received high natural light intensities and, during cloudy weather, supplemental light from fluorescent luminaires. A minimum intensity of 300 ft.-ca. was maintained during the sixteen-hour light period.

The CO_2 concentration was continuously analyzed by an infrared gas analyzer[1] and adjustment of the concentration was made as required. The maximum CO_2 concentration within the chamber was 0.09%; the minimum, 0.009%.

The radioactivity of the air phase was continuously analyzed by an ionization chamber connected with a vibrating diaphragm electrometer. For the first four days, the specific activity of the air phase was adjusted to a high of 2 mc./Gm. C and was not permitted to fall below 1 mc./Gm. C. The specific activity ranged from 1 mc./Gm. C to 0.5 mc./Gm. C throughout the remaining course of the experiment.

The inoculum of *Claviceps purpurea* was prepared from shake-cultures of a sclerotial isolate of the organism. The growth was suspended in distilled water and disintegrated for a few minutes in a Waring Blendor. This suspension was used to inoculate the rye florets. The inoculation was carried out manually, using a hypodermic syringe, for twenty-one days as individual florets began to shed their pollen.

The rye, and the ergot which developed on it, matured within seventy days after having been placed in the chamber. During the course of the experiment, the plants fixed 135.94 mc. of C^{14}.

A total of 704 sclerotia, weighing 64.31 Gm., were isolated from 202 heads of rye; 52 of these heads were not infected. It is of interest to note that of approximately 300 seeds recovered from the rye, none germinated within thirty days of planting.

Extraction and Isolation of the Alkaloids.—The ergot was dried over magnesium perchlorate for five days and then ground with dry ice in a Wiley mill to a 20-mesh powder. The ground ergot had an average absolute activity of 383 μc./Gm. when compared with two ground opium poppy plant standard sources. After drying, the ground ergot was defatted with petroleum ether in a Soxhlet apparatus for seventy-two hours. The defatted ergot, freed of petroleum ether, weighed 58.6 Gm. and had an average absolute activity of 357 μc./Gm.

The ergot, mixed with 3.5 Gm. of $NaHCO_3$ and 24 ml. of water, was allowed to macerate for one hour and then packed in a water-cooled, cylindrical percolator. Washed and ignited sand was used to effect complete transfer.

The ergot was then extracted with 250 ml. of chloroform containing 5% ethanol and the percolate was adsorbed on 25 Gm. of Whatman powdered cellulose. This was reserved for resolution of the three alkaloids.

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† College of Pharmacy, University of Michigan, Ann Arbor.

‡ Division of Biology and Medicine, Argonne National Laboratory, Lemont, Ill.

§ School of Pharmacy, University of Connecticut, Storrs.

[1] Applied Physics Corporation, Pasadena, Calif.

TABLE I.—ANALYSIS OF C^{14}-LABELED ERGOT ALKALOIDS

Alkaloid	Amount Isolated, μg.	Amount Assayed, μg.	Observed c. p. m.	Theoretical c. p. m.[a]	Specific Activity, mc./Gm. C
Ergotoxine	84.0	4.2	531 ± 19.0	17,700	2.8
Ergotamine	546.0	27.3	1826.1 ± 16.3	60,867	1.5
Ergonovine	222.0	11.1	1141.0 ± 7.8	38,033	2.2

[a] Calculated from the per cent efficiency of the counter.

Extraction was then continued until exhaustion, collecting an additional 1.4 L. This percolate was evaporated to dryness under vacuum and the extractive taken up in 50 ml. of ether. Aliquots were assayed colorimetrically and indicated 24 mg. of total alkaloids calculated as ergonovine maleate. The ether was removed from the extract under vacuum and the residue was stored in a freezer.

Following a method similar to that reported by Carless (8), Whatman standard grade powdered cellulose was mixed with McIlvaine buffer, pH 3.0. The mixture was then packed in a glass column 115 × 5 cm. to a height of 80 cm. The cellulose, noted above as containing the extractive of the chloroform-ethanol ergot extract, was mixed with an equal quantity of cellulose. The mixture was transferred to the top of the column and sand was used to facilitate quantitative transfer.

The column was developed at 8–10° with ether saturated with water until 100 ml. had been collected. The column was then developed with ether saturated with water containing 0.1% pyridine. The course of development of the alkaloids was followed by their fluorescence. Experiments with known alkaloids indicated that ergotoxine-complex eluted between 1,850 and 2,200 ml. and that ergotamine eluted between 2,600 and 3,100 ml. After collecting 3,600 ml., 500 ml. of ether saturated with water and containing 1.0% diethylamine was added and, after collecting 500 ml., development was continued with ether saturated with water containing 10% ethanol. Ergonovine had been shown to elute between 1,500 and 1,900 ml. in the latter solvent.

The three eluates were treated with anhydrous Na_2SO_4, filtered, and evaporated to dryness under vacuum. The residues were each dissolved in 1% aqueous citric acid solution and these, in turn, were shaken with ether-chloroform (2:1) mixture.

Aliquots were assayed for radioactivity using a thin, mica endwindow G-M tube and colorimetrically for the amount of alkaloid. Table I shows the results of these analyses.

The radiopurity of the water-insoluble alkaloids was determined by chromatographing 10 μg. of each on Whatman No. 1 filter paper sheets impregnated with citrate-phosphate buffer, pH 4.2, using ether saturated with water as the developing solvent (9). The ergonovine was checked for radioactive contamination by chromatographing 10 μg. on sheets of Whatman No. 1 filter paper and using butanol-acetic acid-water (4:1:5) as the developing solvent (10). The chromatograms were exposed to Kodak No-Screen X-ray film for seventy-two hours and the films then developed.

All three alkaloids were radioactive and proved to have trace quantities of radioactive impurities which rendered them unfit for use in experimental studies.

Attempts at repurification by repeating the column chromatographic separation failed. A modification of this column procedure was attempted. The column was buffered to a pH of 4.2 and anhydrous ether saturated with water was used as the developing solvent. The method was not successful because of incomplete separation of the alkaloids.

Hydrolysis of the Alkaloids and Isolation of Lysergic Acid.—To the extractive representing 24 mg. of total alkaloids which had been stored in a freezer for sixty days, 10.34 mg. of ergotoxine, 10.10 mg. of ergotamine, and 4.93 mg. of ergonovine were added as carriers.

This mixture was dissolved in 50 ml. of 1% aqueous citric acid solution and shaken with nine 25-ml. portions of anhydrous ether to remove non-alkaloidal contaminants. The aqueous layer was adjusted to pH 7 with $NaHCO_3$ and then shaken with four 25-ml. portions of ether-chloroform-ethanol mixture (6:3:1). This extract was dried with anhydrous Na_2SO_4, filtered, and the filtrate evaporated to dryness under vacuum.

The residue was taken up in 20 ml. of ether-chloroform mixture (2:1). One-fourth of this solution was evaporated to dryness and stored in a freezer. Three-fourths was evaporated to dryness and subjected to alkaline hydrolysis and purification by the method of Waldo (11). Aliquots of the product were chromatographed (10) and radiopurity determined as previously described.

The lysergic acid isolated proved to be highly contaminated with radioactive material which ran with the solvent front. Exposure of the chromatograms to X-ray film for ninty-six hours indicated that the lysergic acid isolated was not radioactive.

To determine whether or not the absence of radioactivity was due to decomposition of the alkaloids on storage two aliquots, containing 60 μg. of the reserve mixture of alkaloids, were chromatographed (9) and the chromatograms exposed to X-ray film for seventy-two hours. The results indicated that the alkaloids present were no longer radioactive but contained radioactive material which ran with the solvent front.

DISCUSSION

Biosynthesis of C^{14}-labeled ergot with a specific activity of approximately 1 mc./Gm. C proved successful. Approximately 25 mg. of total alkaloids were isolated from 63 Gm. of ergot. This yield, approximately 0.04%, is unusually small. Whether this is the result of the ionizing radiation, present from the time of inoculation until maturation of the sclerotia, or whether it is the result of biological variation cannot be concluded from the data. It is significant to note that seeds harvested from this same rye failed to germinate.

Resolution of ergonovine, ergotamine, and the

ergotoxine complex was effected by column paper partition chromatography. The specific activities of the alkaloids were unexpectedly high in view of the activity supplied to the plants in the form of CO_2. On checking the radiopurity of the three isolates it was found that trace quantities of radioimpurities were present.

Alkaline hydrolysis of the C^{14} alkaloids (to which carrier alkaloids had been added) to lysergic acid was effected. The lysergic acid isolated proved not to be radioactive. In order to determine whether this apparent loss in radioactivity was the result of decomposition of the alkaloids or whether the radioactivity of the alkaloids resided in the peptide portions of the molecules, aliquots of the mixed alkaloid pool were chromatographed and autoradiographed. The results indicated the alkaloids were not radioactive.

The ergot alkaloids are extremely sensitive to light and heat. Storage at low temperatures and in the dark usually prevents decomposition for many months. The fact that the C^{14}-labeled alkaloids decomposed within sixty days may possibly be the result of the ionizing radiation present. Further experimentation will be required to prove this suggestion.

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Antitumor Activity of Sodium Dichloropyruvate*

By HARRIS BUSCH, SAMIR M. AMER†, and JOSEPH R. DAVIS‡

Sodium dichloropyruvate in doses of 55 to 80 mg./Kg. suppresses growth of the Walker and Jenson rat tumors and prolongs the survival of the tumor-bearing rats.

RECENT STUDIES in this laboratory have indicated that halogenated derivatives of pyruvic acid were potent agents in producing a number of pharmacological and biochemical effects. In the course of these studies, it was found that fluoropyruvic acid was a powerful noncompetitive, partially reversible inhibitor of lactic dehydrogenase (1–3). A variety of analogues of fluoropyruvic acid were prepared, including the mono- and dichloropyruvic acids and the mono- and dibromopyruvic acids (3, 4).

Two points of view led to testing of these compounds as antitumor agents. These compounds bore a resemblance to nitrogen mustards in that they were toxic compounds which exhibited weak cholinomimetic activity. In addition, they were apparently readily absorbed from the blood and inasmuch as the tumor utilizes pyruvate as a hydrogen acceptor, it seemed possible that these compounds might accumulate in the tumor in either the oxidized form of the halopyruvic acid or the reduced form, the halolactic acid.

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† Predoctoral fellow of the University of Alexandria, Egypt.
‡ Postdoctoral fellow of the American Cancer Society.

MATERIALS AND METHODS

The animals used in these experiments were male rats weighing 150–250 Gm. obtained from the Holtzman Rat Co. and fed *ad libitum* on Purina Laboratory Chow.

The Walker tumors were implanted in one subcutaneous abdominal site three to five days before therapy was initiated (5). Solutions for injection were prepared by dissolving the calculated quantity of the drug in triple-distilled water immediately before injection. Sodium dichloropyruvate was prepared by neutralizing the free acid solution with sodium hydroxide to pH 7 prior to injection.

Tumor size was calculated as the product of the three dimensions of the tumor mass measured with the aid of a caliper.

The tumors were measured every other day for nine to ten days after the initiation of therapy. After the tenth day the tumors of the control animals became hemorrhagic and were difficult to measure. The rats were injected with the drug as indicated in Table I throughout the duration of the experiment.

RESULTS

In doses of 55 to 100 mg./Kg., sodium dichloropyruvate inhibited tumor growth by 90.6 to 96.5% (Fig. 1 and Table I) and produced a marked prolongation of the survival time of the tumor bearing rats (Table I). Doses of 70 mg./Kg. every other day of sodium dichloropyruvate produced approximately optimal inhibition of tumor growth and prolongation of survival time. Sodium dichloropyruvate was most effective when admin-

TABLE I.—EFFECTS OF VARYING DOSES AND ROUTES OF ADMINISTRATION OF HALOGENATED PYRUVIC ACID DERIVATIVES ON GROWTH OF WALKER 256 CARCINOSARCOMA AND ON SURVIVAL TIME OF THE TUMOR BEARING RATS

Drug	Dose, mg./Kg.	Route	Repetition	Mean Inhibition, %	Weight Ratio[a]	No. of Animals	Av. Survival Time
Controls	...	..	...	...	1.16	15	11.5
DCPA[b]	42	i. p.	Daily	18.0	1.11	3	17
	55	i. p.	2	83.3	1.01	7	11
	70	i. p.	2	91.7	0.96	7	8
	70	i. p.	3	95.0	1.01	7	11
NaDCP	55	i. p.	2	90.6	1.08	5	17
	70	i. p.	3	93.8	1.15	5	17
	70	i. p.	2	95.2	1.28	5	22
	85	i. p.	2	96.5	1.00	4	23
	100	i. p.	2	91.2	1.03	3	25
FPA	93	i. p.	2	50.0	0.98	2	12
DBPA	15	i. p.	2	54.0	0.94	2	13
	19	i. p.	Once	69.0	0.78	2	4

[a] The change of weight for each animal is indicated as the ratio of the final weight to the initial weight.
[b] DCPA, dichloropyruvic acid; NaDCP, sodium dichloropyruvate; FPA, fluoropyruvic acid; DBPA, dibromopyruvic acid.

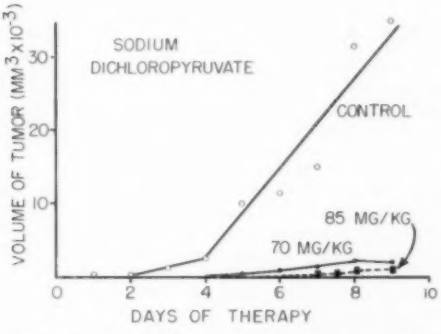

Fig. 1.—The effect of increasing doses of sodium dichloropyruvate on the growth of the Walker 256 carcinosarcoma. The drug was administered intraperitoneally every other day. The tumor size was taken as the product of the three dimensions of the tumor mass as measured with the aid of a caliper.

istered intraperitoneally. The drug was also effective orally in doses of 500 mg./Kg. every two days. Intravenously, the drug seemed to be less active. Despite the continuation of therapy, the tumor became resistant to the drug and ultimately grew to sufficient size to produce emaciation and death.

To determine whether the drug was effective when the tumor was implanted into other sites, the Walker tumor was transplanted into the hamstring muscles and the rats were then treated with 70 to 85 mg./Kg. of sodium dichloropyruvate. Tumor growth was inhibited by 85%[1] with marked prolongation of the survival time.

Rats bearing the Jensen sarcoma implanted into the hamstring muscles were similarly treated and tumor growth was inhibited 67%.

Other Halopyruvic Acids.—Monofluoropyruvic acid was injected into tumor bearing rats in doses of 31 to 93 mg./Kg. Suppression of tumor growth was found only in animals treated with 93 mg./Kg., but the survival time was not prolonged because of the drug toxicity (Table I).

Dibromopyruvic acid exhibited marked antitumor activity in doses of 10 to 20 mg./Kg. However, the toxicity of this drug was so great that the survival times was shortened to only half that of the controls. Neutralization of the dibromopyruvic acid markedly decreased its antitumor activity.

DISCUSSION

These data indicate that sodium dichloropyruvate is carcinostatic in the rat bearing the Walker and Jensen tumors.

While the mode of action of these compounds is uncertain, it is tempting to view them as alkylating agents. Price (6) defined alkylation as the conversion of H-X to R-X. In his view, the first order nucleophilic substitution should involve attachment of the alkyl group (R-) to a group stable as an anion. These requirements may be fulfilled by the halogenated pyruvic acids since the halogen may serve as the stable anion while the rest of the molecule may function as the alkylating group. The halogen is readily lost from these molecules by titration with base (3) and, indeed, Dickens and Williamson have used this cleavage to prepare hydroxypyruvic acid (7). While a stable carbonium ion has not been found, the reactivity of fluoropyruvic acid with glutathione and cysteine has provided evidence for liberation of an alkylating group from halopyruvic acids (8, 9).

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[1] In the case of tumors implanted in the leg, the following formula was used: $V = 4/3\pi r^2 - \pi r_1^2 L$; V is the volume of the tumor mass, r is the radius of the tumor, r_1 is the radius of the leg, and L is the length of the tumor. The formula is based on the assumption that the leg is a cylinder within a spherical tumor mass.

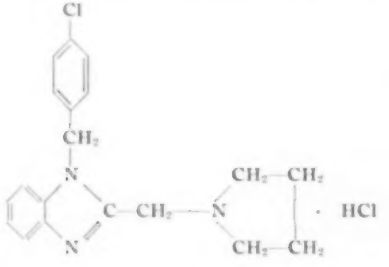
Some Aspects of the Pharmacology of Clemizole Hydrochloride*

By MURRAY FINKELSTEIN, CLARA M. KROMER, SHEILA A. SWEENEY, and CHARLES S. DELAHUNT

Experiments have been described which demonstrate effective prolonged antihistaminic activity and other aspects of the pharmacology of clemizole HCl (1-*p*-chlorobenzyl-2-pyrrolidyl-methyl-benzimidazole hydrochloride). A relatively high degree of antihistaminic specificity was observed in both *in vitro* and *in vivo* experiments. Clemizole was highly effective in antagonizing histamine aerosol bronchospasm. Spontaneous activity of isolated rabbit ileum and intestinal motility in anesthetized dogs were decreased. There was no significant effect on histamine-induced gastric secretion in chronic gastric fistula dogs. Enhanced responses to epinephrine appear to be due to a sensitization to this amine by the antihistamine. Acute and chronic studies showed a relatively low order of toxicity.

THE IMPORTANT ROLE that histamine plays in anaphylaxis continues to stimulate the search for compounds that will antagonize the pharmacological effects of histamine. A new agent, clemizole HCl,[1] 1-*p*-chlorobenzyl-2-pyrrolidyl-methyl-benzimidazole hydrochloride has been recently reported (1–5) capable of antagonizing histamine activity in both *in vitro* and *in vivo* experiments including man. The present investigation was undertaken in order to re-evaluate these observations and to delineate its general pharmacological action with particular emphasis on relative specificity and scope of histamine antagonism.

Clemizole HCl occurs as water-soluble (approximately 2 per cent at 25° C) colorless crystals and has the following chemical structure:



EXPERIMENTAL

Protection against histamine aerosol-induced bronchospasm was determined by a modified Kallos and Pagel (6) technique. Guinea pigs of the Hartley strain (350–400 Gm.) were confined individually in a glass chamber and exposed to a nebulized 0.2% solution of histamine diphosphate. Only animals that suffered asphyxial collapse within two minutes were employed in these experiments. The animals were then exposed to the aerosol at one, three, five, and twenty-four hours following oral drug administration, and at one, two, three, five, and twenty-four hours following intravenous administration. Survival for six minutes of exposure was considered drug protection and is expressed as the per cent of animals protected.

Comparative spasmolytic activity in isolated guinea pig ileum and rat uterus and effects on the spontaneously contracting isolated rabbit ileum were determined by the method previously described (7). The dose required to produce a 50% inhibition of induced spasm (ED_{50}) was estimated from a semilogarithmic plot of dose against the average per cent inhibitory action. The effect on duodenal activity *in vivo* was determined by a closed pressure system in two trained unanesthetized chronic Thiry-Vella fistula dogs (14.5 and 16.8 Kg.), and in four pentobarbital anesthetized dogs (8.9 to 10.2 Kg.). Simultaneous carotid artery blood pressure was recorded in the latter. All compounds were administered intravenously. In the Thiry-Vella dogs each compound was tested on three different days.

The modifying action of clemizole (1 to 10 mg./Kg.) on blood pressure responses to intravenous histamine, acetylcholine, and epinephrine was investigated in a series of eight pentobarbital anesthetized dogs. These were prepared for kymographic recording of blood pressure with a mercury monometer. Respiration was recorded with a Marey tambour connected to a pneumograph fixed to the chest. Similar experiments were performed in four cats prepared in the usual way for the recording of nictitating membrane contractions and blood pressure. Clemizole was administered intravenously in a dose of 3 or 5 mg./Kg. rapidly or over a period of one minute to the dogs and three minutes to the cats. To eliminate the possibility of vagal involvement in blood pressure responses, particularly to epinephrine, the test doses were administered to dogs before and after vagotomy, prior to clemizole administration. Similar experiments were conducted in atropinized dogs. Records of arterial blood pressure, respiration, and Lead II of the electrocardiogram were obtained from an additional series of four pentobarbital anesthetized dogs that received clemizole alone in doses of 1 to 10 mg./Kg.

Three mongrel dogs (11.5 to 16.5 Kg.) with chronic gastric fistulas were employed to determine the effect of clemizole on histamine-induced gastric

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[1] Available under tradename Allercur, J. B. Roerig & Co., Division Chas. Pfizer and Co., Inc.

secretion. Gastric samples (twenty-minute collection periods) were collected for five hours. The first sample following commencement of an experiment was discarded. Two control samples were collected prior to the intramuscular injection of 1 mg. of histamine diphosphate followed by collection of six subsequent samples. Clemizole, 10 mg./Kg., was then administered subcutaneously. Following the next gastric juice sample (twenty minutes) a second dose of histamine was administered and six more samples were collected. Control experiments with the same dogs were conducted according to a similar schedule. Volume, pH, and free acid were determined on each sample. Food was withheld eighteen hours before each experiment.

Acute intravenous and oral toxicity was determined in male Wistar rats and Swiss Webster mice. The LD_{50} and 19/20 confidence limits were calculated according to the method of Litchfield and Wilcoxon (8). The data compiled during a six-month period of chronic toxicity studies with Wistar rats consisted of food consumption and growth rate, organ weights, gross pathology at necropsy, and histological evaluation of tissue sections. Five groups of 15 rats of each sex received clemizole daily in their diet in doses of 10, 30, 100, 300, or 1,000 mg./Kg. In chronic toxicity studies with beagle dogs, additional information was obtained on general appearance, blood chemistry (blood urea nitrogen, alkaline phosphatase, and glucose), liver function (thymol flocculation), complete blood counts, and urinalysis. Four dogs at each dose level received 10, 30, 100, or 300 mg./Kg. daily during the six-month period of observation.

Several comparative studies were conducted with chlorpheniramine maleate (Chlor-Trimeton), its optical *d*-isomer (Polaramine), and tripelennamine hydrochloride (Pyribenzamine). Solutions were always freshly prepared on the day of an experiment.

RESULTS

Histamine-Aerosol Bronchospasm.—The summarized data in Table I obtained from two series of experiments show that clemizole effectively protected guinea pigs against a challenge of histamine aerosol. Onset of activity was relatively rapid. The number of animals protected and the duration of protective action were dependent on the dose. In comparative evaluations clemizole was more effective than tripelennamine and essentially similar to chlorpheniramine in these experiments (Table I). No protection was observed twenty-four hours following administration of the compounds.

In Vitro Relative Spasmolytic Activity.—The summarized data of two series of experiments presented in Table II show that tripelennamine, chlorpheniramine, and *d*-chlorpheniramine were approximately 40, 10, and 14 times, respectively, as potent as clemizole in inhibiting histamine-induced ileal contractions. The anticholinergic activity of clemizole was extremely low but similar to that of tripelennamine. It was relatively high in musculotrophic action and approximately one-third as potent as tripelennamine in antiserotonin action. The antihistaminic activity of clemizole was prolonged, particularly with the higher concentrations, as evidenced by the absence of complete recovery of histamine contractions following blockade. This suggests that clemizole may become "fixed" in the tissues in some instances, thus prolonging antihistaminic activity.

Clemizole concentrations of 1 to 10 mcg./cc. produced a diminished tonus and a decrease in amplitude and rate of spontaneous contractions of isolated rabbit ileum (Fig. 1). Repeated washing resulted in almost complete recovery. Whereas tripelennamine showed greater antihistaminic activity than clemizole (Table II), it was less potent than clemizole in suppressing rabbit ileum activity (Fig. 1).

In Vivo Intestinal Motility.—*Unanesthetized Chronic Thiry-Vella Fistula Dogs.*—With one exception, clemizole, 3 mg./Kg., was without effect on intestinal motility and tone. In a single experiment which was not duplicated with the same dog there was a slight transient increase in tone and amplitude of contraction. A similar dose of tripelennamine usually produced no effect but in two instances which were not duplicated there was a decrease in tone and motility.

Anesthetized Dogs.—As can be seen in Fig. 2, both clemizole and tripelennamine at 3 mg./Kg. were effective in diminishing tonus and depressing motility. This effect was present immediately upon completion of injections and the duration of action was thirteen to twenty-five minutes. A higher dose of clemizole, 10 mg./Kg., usually produced similar results. However, in one experiment this dose induced a transient increase in tone and amplitude of contraction. There is no ready explanation for the difference between the present observations and those of Sherrod, *et al.* (9), who showed that tripelennamine increased duodenal tone in similar preparations. The effect on blood pressure seen in Fig. 2 is more pronounced than that seen in Fig. 3. The experimental preparation, in addition to the rapid rate of injection in this animal were undoubtedly contributory factors.

Gastric Secretion.—Complete data are presented in Table III. At first glance it would appear that clemizole produced a slight inhibition of histamine-induced gastric flow. However, on close examination it becomes apparent, indeed, that the volume of secretion and output of free acid were not significantly affected by drug action. There was, particularly in dog No. 3, a tendency toward reduced secretion after drug injection. A suggestion of this tendency is also observed in the average results. However, control experiments also exhibited a decreased response to the second injection of histamine. In addition, considerable inconsistency was observed in dog No. 2.

These results suggest that there was no specific antagonism between clemizole and histamine in respect to gastric secretion. This view is supported when one considers the relatively little, if any, effect observed in this experiment as compared to the large degree of inhibition of other physiological actions of histamine. It appears that clemizole produces less inhibition on the gastric gland cells than other cells activitated by histamine, as is undoubtedly the case with other known antihistaminics.

Cardiovascular.—In dog experiments clemizole usually produced a slight initial transient fall in blood pressure (5 to 24 mm. Hg) which was often followed by a similar degree of transient or pro-

TABLE I.—PROTECTION AGAINST HISTAMINE AEROSOL-INDUCED BRONCHOSPASM IN GUINEA PIGS FOLLOWING ORAL AND INTRAVENOUS DRUG ADMINISTRATION

Compound and Route	Dose mg./Kg.	No. of Animals	Per Cent Protected at 1 hr.	2 hr.	3 hr.	5 hr.
Series A						
Clemizole, i. v.	0.25	8	62	25	12	0
	0.50	8	100	100	75	50
	1.0	8	100	75	75	38
	2.0	8	100	100	100	50
	4.0	8	100	100	100	88
Tripelennamine, i. v.	0.25	8	12	25	0	0
	0.50	8	50	25	0	0
	1.0	8	88	62	38	0
	2.0	8	100	100	38	0
	4.0	8	100	100	50	0
Clemizole, oral	1	8	50	..	25	0
	4	8	88	..	62	50
	8	8	100	..	100	88
	16	8	100	..	100	100
Tripelennamine, oral	1	8	12	..	0	0
	4	8	25	..	12	12
	8	8	38	..	38	25
	16	8	75	..	38	12
Series B						
Clemizole, i. v.	0.25	9	33	33	22	22
	0.50	9	89	33	22	22
Chlorpheniramine, i. v.	0.25	9	67	33	11	11
	0.50	9	89	78	44	11
Clemizole, oral	1.0	6	67	..	67	67
	2.0	6	67	..	67	33
	4.0	6	100	..	100	83
	8.0	6	100	..	100	100
Chlorpheniramine, oral	1.0	6	83	..	67	17
	2.0	6	100	..	67	17
	4.0	6	100	..	100	50
	8.0	6	100	..	100	100

TABLE II.—COMPARATIVE SPASMOLYTIC ACTIVITY (APPROXIMATE ED_{50})

Compound	ED_{50} Against Spasms Induced By[a]: Histamine (Guinea Pig Ileum)	Acetylcholine (Guinea Pig Ileum)	$BaCl_2$ (Guinea Pig Ileum)	Serotonin (Rat Uterus)
Series A				
Clemizole	0.021	4.4	0.58	3.3
Tripelennamine	0.0005	5.2	6.4	1.2
Atropine	...	0.0025	..	..
Series B				
Clemizole	0.013	..	..	..
Chlorpheniramine	0.0014	..	..	..
d-Chlorpheniramine	0.00093	..	..	..

[a] Concentration of spasmogens: histamine 0.25 mcg./cc.; acetylcholine 0.25 mcg./cc.; barium chloride 100 mcg./cc.; serotonin 2.5 mcg./cc.

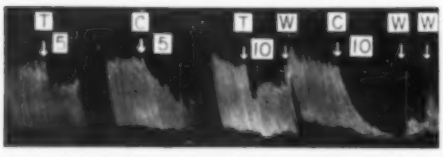

Fig. 1.—Effect of clemizole (*C*) and tripelennamine (*T*) on spontaneous activity of isolated rabbit ileum. Doses of 5 and 10 are in mcg./cc. *W* = wash.

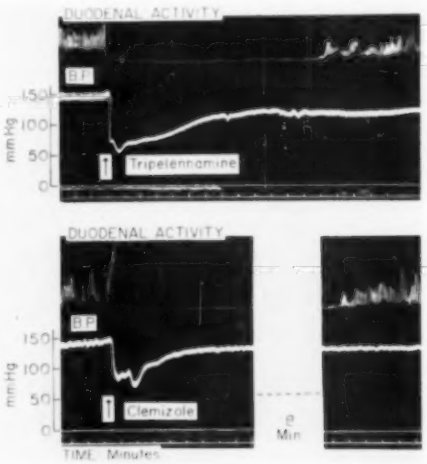

Fig. 2.—Effect of intravenous clemizole and tripelennamine 3 mg./Kg. on duodenal activity and blood pressure of anesthetized dog. Rapid drug injection.

longed rise. However, the degree of fall in pressure was directly proportional to the rate of injection as seen on a comparison of Figs. 2 and 3. In the cat experiments where the total clemizole dose (3 mg./Kg.) was administered in three minutes in con-

TABLE III.—EFFECT OF CLEMIZOLE ON GASTRIC SECRETION

	Dog No.	Collection Period[a] 1	2	H[b]	Collection Period[a] 1	2	3	4	5	6	Coll. Per.[a] C[c]	1	H[b]	Collection Period[a] 1	2	3	4	5	6
									Volume, cc.										
Controls	1	18.8	16.0		23.1	26.3	13.0	4.7	5.0	2.5	2.8		5.2	22.0	4.2	4.5	5.7	3.3	
	2	1.0	2.7		16.0	22.1	5.5	3.0	2.0	2.0	1.5		11.0	29.0	7.5	4.8	3.3	2.0	
	3	15.0	13.0		20.1	38.0	14.2	2.0	4.5	6.0	6.0		7.5	24.0	5.0	6.0	8.5	2.0	
Average		11.6	10.6		19.7	28.8	10.9	3.2	3.8	3.5	3.1		7.9	25.0	5.6	5.1	5.8	2.4	
Clemizole,	1	35.0	28.1		52.5	40.1	7.3	2.0	<1	<1	<1		11.2	11.9	7.3	2.0	<1	<1	
10 mg./Kg.,	2	20.7	15.0		43.5	62.0	24.5	13.5	3.8	1.5	1.3		12.0	26.0	16.0	9.5	6.0	3.1	
subcutane-	2	2.8	2.0		10.0	16.6	7.4	3.2	1.5	2.0	1.0		12.0	21.0	9.0	7.0	3.0	3.3	
ously	3	2.5	21.0		76.0	48.5	16.4	2.3	2.5	6.5	3.5		3.0	19.9	18.0	11.0	9.5	2.0	
Average		15.2	16.5		45.5	41.8	13.9	5.2	2.6	3.3	1.9		9.6	19.7	12.6	7.4	6.2	2.8	
									Free Acid, meq./100 cc.										
Controls	1	0	0.10		0.21	1.06	0.85	0.41	0	0	0		0.10	2.75	1.69	1.48	0.42	0	
	2	0.74	0		6.35	13.75	12.27	5.71	5.08	4.23	0.64		4.23	11.21	12.91	12.27	5.08	5.08	
	3	0	0		2.33	11.43	11.00	5.08	0.85	0.42	0		1.90	7.62	9.31	5.50	1.06	1.70	
Average		0.25	0.03		2.96	8.75	8.04	3.73	2.96	2.32	0.32		2.08	7.19	7.97	6.42	2.19	3.39	
Clemizole,	1	9.95	10.16		11.21	11.21	7.83	4.23	[d]	[d]	[d]		1.06	7.83	6.98	2.96	[d]	[d]	
10 mg./Kg.,	2	14.39	14.39		13.54	13.12	6.77	11.00	11.43	6.77	5.07		5.92	11.00	12.91	14.18	8.89	8.46	
subcutane-	2	3.81	1.27		1.06	11.64	7.41	5.08	6.35	5.93	4.22		5.50	11.43	12.91	10.37	5.93	3.81	
ously	3	0	0		4.66	13.75	13.54	8.25	5.93	0	0		0	6.56	8.46	5.92	5.50	3.38	
Average		7.04	6.46		7.62	12.43	8.89	7.14	7.90	4.23	3.10		3.12	9.20	10.32	8.36	6.77	5.22	

[a] Collection period equals twenty minutes. [b] Histamine—1 mg. intramuscularly. [c] Clemizole—10 mg./Kg. orally. [d] Insufficient sample for assay.

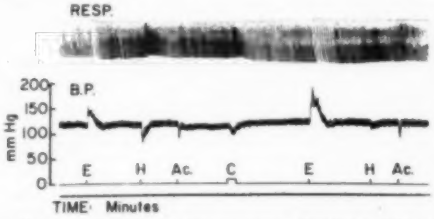

Fig. 3.—Effect of intravenous clemizole (*C*), 3 mg./Kg., on blood pressure and respiration of pentobarbital sodium anesthetized dog, and influence on pressure response to intravenous epinephrine 5 mcg./Kg.(*E*), histamine 5 mcg./Kg. (*H*), and acetylcholine 10 mcg./Kg.(*Ac.*). Rate of clemizole injection = total dose in one minute.

trast to one minute in the dogs, there was essentially no change in blood pressure. The rate of respiration was temporarily increased and there were no significant changes in the electrocardiogram. The heart rate decreased temporarily by 10 to 20 beats per minute in 50% of the dogs and no changes were observed in the remaining animals. The decreased heart rate which was observed occasionally appeared to occur primarily following the more rapid administration of clemizole. It is likely that this effect may have been due to an acute transient depressed myocardium. In preliminary experiments (not reported here) with the isolated cat heart papillary muscle it was observed that only in the presence of relatively high concentrations of clemizole was there a decrease in contractile force (approximately 10% at 20 mcg./cc. and 20% at 40 mcg./cc.). Tripelennamine produced similar effects. The transient fall in blood pressure seen with both drugs may have also been due to this action.

The modifying action of clemizole on blood pressure responses to intravenous histamine, acetylcholine, and epinephrine may be seen in a typical experiment shown in Fig. 3. The depressor response to histamine following clemizole at a dose of 3 mg./Kg. was diminished by an average of approximately 50% with a range of 20 to 95%. This dose and also that of 5 mg./Kg. did not antagonize the depressor response to acetylcholine.

The pressor response to epinephrine following the administration of clemizole was enhanced by an average of approximately 30% with a range of 10 to 75%. Contraction of the cat nictitating membrane was also increased. It appears that the augmented pressor response was not due to an inhibition of cardiac vagal impulses or to an atropine-like action. The epinephrine pressor response was essentially unaltered in experiments where the vagi had been cut or where the animals were atropinized. It is very likely that this phenomenon is due to a sensitization of the myocardium and/or the arterioles to epinephrine, and possibly also a sensitization of other tissues that respond to administered epinephrine. This has been previously alluded to by Sherrod, *et al.* (9), and by Yonkman, *et al.* (12), in their studies on tripelennamine. In the preliminary experiments with isolated cat heart papillary muscles mentioned above, it was observed that the positive inotropic effect induced by epinephrine was augmented in the presence of a clemizole or tripelennamine concentration of 10 mcg./cc. This concentration of the antihistaminics produced no effect on the contractile force. In another series of preliminary experiments it was noted that whereas intravenous clemizole (dose of 3 mg./Kg. administered over period of three minutes) was essentially without effect on the blood pressure of pithed cats it produced a relatively sharp augmentation of the maintained pressor response to infused epinephrine. These observations tend to support the views expressed above.

Acute Toxicity.—The data, LD_{50}, obtained with clemizole and a comparison with those of other antihistaminics described in the literature are summarized in Table IV. It may be seen that clemizole possesses a relatively low order of toxicity.

Chronic Toxicity.—The only toxic effect observed

TABLE IV.—COMPARATIVE ACUTE TOXICITIES, LD_{50}, IN MICE AND RATS

Compound	Species	Route	LD_{50} mg./Kg.[a]	
Clemizole	Mouse	Oral	837	(775–904)
		i. v.	75	(67–84)
	Rat	Oral	1950	(1773–2145)
		i. v.	74	(65–85)
Tripelennamine[b]	Mouse	Oral	210	
		i. v.	12	
	Rat	Oral	570	
		i. v.	12	
Chlorpheniramine[c]	Mouse	Oral	121	(102–143)
		i. p.	73	(60–68)
		i. v.	20	(14–29)
	Rat	Oral	118	(95–148)
		i. p.	89	(73–108)

[a] With 19/20 confidence limits. [b] Data of Mayer, *et al.* (10). [c] Data of Roth and Govier (11).

in rats was at the highest dose, 1,000 mg./Kg., in the form of severe growth retardation. The hematological and histological findings were essentially normal at all doses. The following toxic symptoms, which are typical for most antihistaminics, were manifested in dogs receiving the higher dose of clemizole: anorexia, weight loss, emesis, and muscle tremors. The general physical conditions along with the hematological, biochemical, and histological findings for the six-month period were all within the normal limits of variation.

SUMMARY AND CONCLUSIONS

1. Experiments have been described which demonstrate effective, prolonged antihistaminic activity and other aspects of the pharmacology of clemizole HCl.

2. A relatively high degree of antihistaminic specificity was observed in both *in vitro* and *in vivo* experiments.

3. Clemizole was more potent than tripelennamine and similar to chlorpheniramine in protecting against histamine aerosol-induced bronchospasm *in vivo*, but weaker than either compound in blocking histamine contractions of isolated guinea pig ileum.

4. Clemizole decreased spontaneous activity of isolated rabbit ileum and showed a similar effect on intestinal motility in anesthetized dogs but produced essentially no effect in unanesthetized chronic Thiry-Vella dogs. It was also effective in antagonizing the spasmogenic action of barium chloride in isolated intestinal strips.

5. There was no significant effect on histamine-induced gastric secretion in chronic gastric fistula dogs.

6. Intravenous administration produced a transient effect on blood pressure, the degree and duration of which were directly related to rate of administration. There was little, if any, change in heart rate, no effect on the electrocardiogram, and a transient increase in rate of respiration.

7. Clemizole produced enhanced responses to epinephrine which may be due to a sensitization to this amine.

8. Acute and chronic studies showed a relatively low order of toxicity.

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An Investigation of the Culture, Constituents, and Physiological Activity of *Panaeolus campanulatus**

By V. E. TYLER, Jr., and M. H. MALONE

Wild-growing and cultivated carpophores of *Panaeolus campanulatus* were found to contain serotonin, 5-hydroxytryptophan, tryptophan, urea, and citrulline, but none of these compounds was present in the mycelium or nutrient medium obtained from cultures of the organism. Five additional indole derivatives were detected in the carpophores. These remain unidentified although they were shown not to be psilocybine, tryptamine, or N,N-dimethyltryptamine. An extract of the mushroom was found to exhibit parasympathomimetic activity which was muscarinic in nature. Choline was identified chromatographically, but muscarine, which was shown physiologically to exist in minute amounts in an extract, could not be detected by the methods employed. After physiological tests in small animals revealed that the mushroom was relatively nontoxic, a quantity was ingested by one of the investigators to test its reported hallucinogenic activity. No effects were noted, and it is concluded that this fungus does not produce cerebral mycetism nor is it a dangerously poisonous mushroom.

CONSIDERABLE CONFUSION centers about the black-spored agaric commonly referred to as *Panaeolus campanulatus* (Fr.) Quélet. Even its proper scientific name is in dispute. Singer and Smith (1) have concluded that if the species is considered conspecific with *Panaeolus sphinctrinus* (Fr.) Quélet, it should be known under that name, and if not, as *Panaeolus linnaenus* Imai. For reasons which will be advanced later in this paper it would appear that the latter name should be adopted.

The physiological activity of *P. campanulatus* is also controversial. Several cases of cerebral mycetism produced by members of the genus have been reviewed by Heim (2). Of these cases one, which was reported in 1816, was attributed to *Agaricus* (*Panaeolus*) *campanulatus*, but at this time it cannot be ascertained if the species identification was correct. Singer (3) has pointed out that members of this genus are not determinable by the layman and only with difficulty by the specialist.

Availability of a quantity of authentic *Panaeolus campanulatus* prompted an investigation of some of the chemical and physiological properties of the mushroom in an attempt to clarify some of these problems. Since the fungus had been shown previously (4) to contain a number of indole derivatives, including serotonin, a pure culture of the organism was prepared and investigated with respect to its ability to biosynthesize derivatives of the indole nucleus.

EXPERIMENTAL

Collection of Mushrooms.—Specimens of the mushroom were collected during the spring of 1958 near Olympia, Washington and were identified as *Panaeolus campanulatus* (Fr.) Quélet by Prof. D. E. Stuntz, Department of Botany, University of Washington. A culture was prepared from one of the fresh carpophores by carefully breaking open the pileus and removing a bit of the sterile, internal tissue with a mycologist's spade. The tissue fragment was transferred to a culture tube containing a slant of dung agar medium (5) where it began to produce typical mycelial strands within a few days. Some of the remaining carpophores were dried in a circulating-air oven at 50°, others were preserved by immersion in 95% ethanol.

Cultivation and Extraction.—In order to determine if the organism produced serotonin and related indole compounds in its mycelial state, four Roux-type culture bottles, each containing 125 ml. of the liquid medium of Heim, *et al.* (6), modified by the substitution of 4.5% malt extract for the beer wort, were prepared. The flasks were inoculated and placed in a constant temperature cabinet, which admitted subdued artificial light through its glass doors, and maintained at 25 ± 1° for a period of thirty days. Under these conditions the fungus underwent moderately rapid mycelial development, but no fruiting bodies were produced. At the conclusion of the incubation period, the mycelial mats were removed from the flasks, dried in a circulating-air oven at 50°, and weighed. The average dry weight of mycelium was 1.43 Gm. per flask.

The dried mycelium was reduced to a No. 60 powder in a Wiley laboratory mill, and a 3-Gm. quantity was placed in a test tube together with 15 ml. of 70% ethanol and agitated vigorously on a mechanical shaker for two hours. At the conclusion of this period the tube was centrifuged, and the extract decanted and allowed to evaporate to dryness at room temperature. The residue was redissolved in 2 ml. of 70% ethanol, and 60-μl. quantities of this solution were subjected to chromatographic analysis according to the procedure subsequently described.

The growth medium was decanted from the flasks, measured, and found to average 82 ml. per flask. The culture liquids of all the flasks were combined and reduced to dryness under reduced pressure in a Flash-Evaporator at 50°. This residue was then re-extracted with 100 ml. of 70% ethanol and the resulting extract filtered and evaporated as before to a vol-

* Received July 9, 1959, from the College of Pharmacy, University of Washington, Seattle and the College of Pharmacy, University of New Mexico, Albuquerque.

ume of 8 ml. Forty-microliter portions of this extract were then analyzed chromatographically.

To simulate more nearly in the laboratory the natural growth conditions of the fungus, a quantity of fresh horse dung, together with a little tap water, was placed in each of twelve 500-ml. Erlenmeyer flasks. After autoclaving, the flasks were inoculated and placed in the incubator at 25° where mycelial development took place and soon permeated the solid medium. After three weeks, five of the flasks were removed from the incubator and placed in a greenhouse where they were exposed to direct sunlight for several hours daily, as well as to diurnal temperature variations ranging between 15 and 35°. In less than two weeks normal carpophore development was noted in some of the flasks, and within four weeks all five showed fruiting body formation. These mushrooms were normal in every respect with the exception of slightly elongated stems (see Fig. 1). They were harvested periodically, counted, dried, and weighed, with the results recorded in Table I. The flasks continued to produce during a total period of fifteen weeks or twelve weeks after placement in the greenhouse.

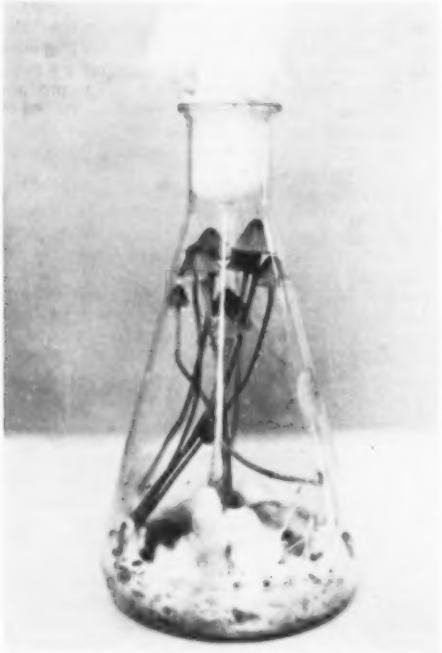

Fig. 1.—Cultivated carpophores of *Panaeolus campanulatus* in 500-ml. Erlenmeyer flask.

Of the seven flasks remaining in the incubator, none showed carpophore formation nine weeks after inoculation. In order to determine if the production of fruiting bodies was dependent on sunlight or was influenced merely by the temperature fluctuations, six of these remaining flasks were taken at the end of nine weeks and placed in the greenhouse; three in direct sunlight and three in a light-tight box. Two of the three flasks placed in the sunlight developed

TABLE I.—PRODUCTION OF CARPOPHORES OF *Panaeolus campanulatus* IN STERILE CULTURE

Flask No.	No. Carpophores	Fresh Wt., Gm.
1st Collection (6th Week of Growth)		
1	6	0.72
2	7	0.96
3	5	0.48
4	..	..
5	..	..
2nd Collection (8th Week of Growth)		
1	7	2.18
2	11	5.05
3	5	1.69
4	5	3.00
5	3	0.42
3rd Collection (12th Week of Growth)		
1	6	2.04
2	3	5.05
3	5	0.80
4	1	0.50
5	2	0.90
4th Collection (15th Week of Growth)		
1	4	3.00
2	1	0.67
3	10	4.55
4	2	1.30
5	3	1.03
Totals		
1	23	7.94
2	22	7.06
3	25	7.52
4	8	4.80
5	8	2.35
Av.	17	5.93

carpophores within two weeks. None of the cultures subjected to diurnal temperature variations in the absence of light produced carpophores during the six week period following their removal from the incubator. The single flask remaining in the incubator also failed to produce fruiting bodies during the entire course of the experiment.

Quantities of the dried carpophores, both cultivated and naturally occurring, were milled to a No. 60 powder and 0.5-Gm. quantities of each were extracted with 15 ml. of 70% ethanol as previously described. In this case, however, the final residues were each dissolved in 1 ml. of 70% ethanol and 40-μl. portions were subjected to chromatographic analysis.

Chromatographic Analysis.—Because of the large number of indole derivatives detected during the course of a preliminary investigation of *Panaeolus campanulatus* (4), it was necessary to utilize a two-dimensional paper chromatographic technique for satisfactory separation of all of these compounds. Investigation of a number of different acidic, neutral, and basic solvent systems revealed that effective separation was obtained when a mixture of *n*-propanol-1 *N* ammonia (5:1) was employed as the first wash liquid, followed by *n*-butanol-acetic acid-water (4:1:5) in the second direction. Sheets of Whatman No. 1 filter paper, $18^1/_4 \times 22^1/_2$ in., were utilized, and the direction of formation was ascending; time averaged twenty-two hours in each direction. The various extracts were spotted in replicate 10-μl. portions, each being dried before addition of the next, until the specified amount was applied. After

formation, the sheets were sprayed with 2% *p*-dimethylaminobenzaldehyde in 1 *N* hydrochloric acid (7) or with Pauly's reagent (8). Identification of the spots was based on comparison with colors and locations of reference compounds run as single compounds and in mixture with each other and with the various extracts. Average R_f values of the principal indole and urea derivatives, identified and unidentified, are recorded in Table II. Since these values vary slightly with the identity of the other components in the mixture and with concentration, these figures should be considered as indicative of the relative degree of separation achieved rather than absolute values.

TABLE II.—CHROMATOGRAPHIC DATA FROM EXTRACTS OF *Panaeolus campanulatus*

R_f[a] Values × 10² Wild Carpophores	Cultured Carpophores	Color Reaction With *p*-Dimethylaminobenzaldehyde	Identity of Compound
3/15	2/13	Yellow	Citrulline
0/19	0/18	Bluish (faint)	?
6/25	4/24	Yellow	?
11/26	8/24	Purple	5-Hydroxytryptophan
17/39	17/35	Bluish (very faint)	?
20/46	20/46	Reddish violet	Tryptophan
17/78	17/79	Bluish (very faint)	?
28/79	30/79	Bluish	?
38/44	38/47	Yellow	Urea
53/49	49/51	Purple	Serotonin

[a] Expressed as: R_f obtained with *n*-propanol-ammonia/R_f obtained with *n*-butanol-acetic acid-water.

After the pharmacological investigation had revealed the presence of a compound(s) exhibiting parasympathomimetic activity in this mushroom, quantities of an extract prepared in the manner described in the section on pharmacological evaluation were examined chromatographically for the presence of choline, acetyl choline, and muscarine. A solution of the extract was spotted on sheets of Whatman No. 1 filter paper and formed in an ascending direction for twenty-four hours with a wash liquid composed of butanol-ethanol-water (5:5:2). Kögl, *et al.* (9), report that in this solvent system choline exhibits an R_f of 0.37, acetylcholine 0.44, and muscarine 0.50. The chromatograms were evaluated with Thies and Reuther's reagent which Eugster (10) has reported is capable of detecting as little as 6 mcg. of muscarine.

When quantities of extract representing approximately 0.25 Gm. of fresh mushrooms were analyzed by this procedure, neither acetyl choline nor muscarine was observed, but a well-defined spot was noted with an R_f value averaging 0.36. This spot had the same R_f value as a control spot of known choline and did not separate from that reference compound in a mixed chromatogram. Although no muscarine was detected, calculation revealed the quantity found to be present by physiological methods in this amount of extract would be below the sensitivity of the chromatographic detection method employed.

Attempts to chromatograph quantities of the crude extract representing quantities of fresh mushrooms as large as 5 Gm. were made. On the basis of physiological tests, this quantity of plant material should contain approximately 65 mcg. of muscarine, a quantity readily detectable with Thies and Reuther's reagent. However, at this concentration, the relatively large amount of choline and other impurities in the extract produced a distortion of R_f values, and the presence of muscarine could not be verified.

Pharmacological Investigation.—For the preliminary physiological testing in small animals an extract was prepared from a 155-Gm. quantity of fresh *Panaeolus campanulatus* which had been preserved in 95% ethanol. The mushrooms and the alcohol (approximately 200 ml.) were homogenized in a Waring Blendor for two minutes and the cellular debris separated from the extract by filtration on a Büchner funnel. The marc was then returned to the Blendor vessel and re-extracted, first with 250 ml. of 70% ethanol for three minutes, then with two successive 325-ml. portions for five minutes each. The successively collected filtrates were combined and evaporated to a syrupy residue under reduced pressure in a Flash-Evaporator at 50°.

The residue was taken up in two successive 100-ml. portions of distilled water; the extracts combined, filtered, and again reduced to a solid residue in the Flash-Evaporator. The soluble portion of the residue was dissolved in a series of small volumes of distilled water (total 25 ml.) which were combined, evaporated, and the residue dried in a desiccator over calcium chloride. The solid extract weighing 2.84 Gm. and the dried marc from the alcoholic extractions (8.25 Gm.) were tested for gross physiological activity in rats.

Rats were previously unused twelve-week-old male animals of the Wistar strain, fasted overnight prior to testing. All injections were made intraperitoneally into the upper left quadrant of the abdomen after the rats had been weighed to the nearest whole gram. Dosage volume for all injections was 5 ml./Kg. Solutions of the extract were made utilizing distilled water, while solution-suspensions of the marc were prepared using 0.25% agar solution.

Injections of the marc produced no physiological effects up to 500 mg./Kg., which was the maximum dosage physically capable of being injected. At this peak dosage level, some ptosis, respiratory depression and abdominal tenderness were noted.

Injections of the extract were physiologically inert up to 1 Gm./Kg. at which level lacrimation, micturition, bloody tears, decrease in respiratory rate, increase in respiratory depth, and reduction of motor activity were noted. Maximal response was seen one to two hours after injection. These symptoms were all indicative of parasympathomimetic activity. No miosis was seen.

All animals receiving the extract and the marc were observed for two weeks after the injections. No delayed toxicity was observed.

Utilizing a modified Magnus technique (11), the extract was further screened using isolated rabbit intestine. Perfusion medium used was oxygenated Sollmann-Radamaeker's solution maintained at 37°; bath size was 100 ml. Rabbits were killed by a sharp blow at the back of the neck. Extract bath concentrations of 1:50,000 produced a submaximal increase of intestinal tone with little to no effect on peristaltic rate. A concentration of 1:2,500 had a depressant effect on tone and peristalsis.

In the absence of a sample of muscarine, methacholine chloride was used as a reference compound, as methacholine has been shown to be primarily muscarinic in action with little to no nicotinic activity (12). Concentrations of 1:100,000 methacholine chloride and 1:50,000 of the extract are roughly comparable qualitatively and quantitatively (see Figs. 2 and 3). A bath concentration of 1:10,000 atropine sulfate effectively blocked methacholine, but a bath concentration of 1:2,500 was required to comparably block the extract. The addition of the extract (1:5,000) to the bath containing methacholine partially reversed the atropine atony of the intestine refractory to methacholine. Raising the concentration of the extract from 1:50,000 to 1:4,500 partially reversed the atropine blockade of the extract. The ability of the extract to reverse the parasympatholytic effects of atropine is further illustrated in Fig. 4. Atropine sulfate, 1:10,000, effectively blocked methacholine chloride response at bath concentrations of 1:33,000, but was reversed by 1:2,500 concentration of the extract. Further addition of atropine, in turn, reversed the stimulation of the extract, indicating a competitive inhibition between these two agents. It has been noted that if muscarine and atropine are administered concurrently or successively, their respective quantities determine which effect predominates, although atropine is the stronger of the two (13).

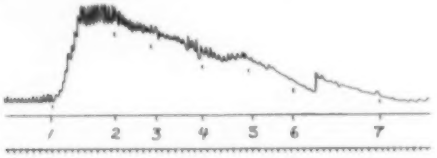

Fig. 2.—Additions to 100-ml. bath. *1*, 2 mg. extract; *2*, 10 mg. atropine sulfate; *3*, 10 mg. atropine sulfate; *4*, 10 mg. atropine sulfate; *5*, 10 mg. atropine sulfate; *6*, 20 mg. extract; *7*, 200 mg. extract.

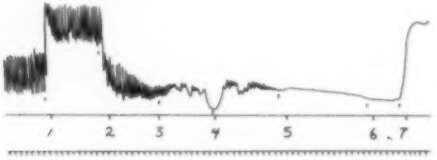

Fig. 3.—Additions to 100 ml. bath. *1*, 1 mg. methacholine chloride; *2*, 10 mg. atropine sulfate; *3*, 2 mg. methacholine chloride; *4*, 20 mg. extract; *5*, 2 mg. methacholine chloride; *6*, 2 mg. methacholine chloride; *7*, 10 mg. barium chloride.

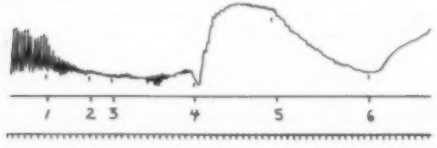

Fig. 4.—Additions to 100 ml. bath. *1*, 10 mg. atropine sulfate; *2*, 1 mg. methacholine chloride; *3*, 2 mg. methacholine chloride; *4*, 40 mg. extract; *5*, 20 mg. atropine sulfate; *6*, 10 mg. barium chloride.

Qualitatively the extract exhibits parasympathomimetic activity that is muscarinic in nature. Similar techniques utilizing isolated rabbit intestine have shown that muscarine produced submaximal effects at a bath concentration of 1:67,000,000 (14). Assuming that the activity of this extract is due to muscarine alone, the content of this extract would be approximately 0.07% and that of the fresh mushrooms approximately 0.0013%.

After its detection by chromatographic analysis, choline chloride was tested on isolated rabbit intestine. Bath concentrations of 1:100 produced submaximal effects comparable to those seen previously with methacholine and the extract (see Fig. 5). Acetylcholine chloride was also tested for reference purposes and produced submaximal effects at a concentration of 1:100,000. Complete blockade of choline and acetylcholine was effected by 1:10,000 concentrations of atropine sulfate. Further additions of choline and acetylcholine did not reverse the atropine atony. Assuming that the activity of the extract is due solely to choline, the choline content was calculated to be 50,000%, an impossible figure. The collected data indicate, therefore, that while the choline found in the extract contributed to the parasympathomimetic effect, there was a more powerful agent also present. Assuming that the major portion of the extract is choline, and that the remaining parasympathomimetic activity is due solely to muscarine, calculations still indicate about 0.07% of muscarine in the extract. The gross parasympathomimetic effects seen in the intact rat dosed with 1.0 Gm./Kg. of the extract cannot be produced by dosage levels of choline that could be physically present.

After the preliminary tests in rats had indicated that *Panaeolus campanulatus* was relatively nontoxic but possessed a certain amount of physiological activity, it was considered necessary to test the activity of the mushroom in man, since drugs influencing the higher brain centers in human beings do not always demonstrate their typical effects in lower animals. Six dried carpophores of the mushroom (dry weight, 0.8 Gm.) were ingested by one of us (V. E. T., Jr). Initially, two of the mushrooms were thoroughly masticated and swallowed. When no effects were observed after thirty minutes, four more carpophores were ingested. The mushrooms proved to be nearly tasteless at first but developed a slightly acrid, mucilaginous taste on chewing, which was considered to be somewhat unpleasant. No physiological effects of any kind were observed following the ingestion of this quantity of the mushroom.

RESULTS AND CONCLUSIONS

No qualitative differences were detected chromatographically in the composition of the wild and the cultivated carpophores of *Panaeolus campanulatus*. Serotonin, 5-hydroxytryptophan, tryptophan, urea, and citrulline were identified as constituents of both types of fruiting bodies, but none of these compounds was detected in the mycelium or the medium obtained from mycelial cultures of the organism. Identification of these compounds was based on R_f values and color reactions with *p*-dimethylaminobenzaldehyde. In

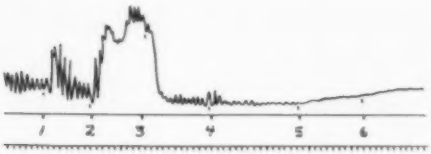

Fig. 5.—Additions to 100 ml. bath. *1*, 100 mg. choline chloride; *2*, 900 mg. choline chloride; *3*, 10 mg. atropine sulfate; *4*, 1,000 mg. choline chloride; *5*, 1,000 mg. choline chloride; *6*, 1,000 mg. choline chloride.

addition, the serotonin and 5-hydroxytryptophan exhibited characteristic pinkish colors after being subjected to Pauly's reaction.

The occurrence of 5-hydroxytryptophan is not surprising in view of the presence of serotonin in the mushroom and the fact that 5-hydroxytryptophan has been shown to serve as a precursor to serotonin, at least in animal tissue (15). The presence of both compounds here is indicative of a similar biosynthetic pathway in this fungus.

On the basis of the typical color reactions with *p*-dimethylaminobenzaldehyde, it was concluded that five additional unidentified indole derivatives and one other unidentified urea derivative were present in the carpophores. Comparison with the color reactions and R_f values of reference compounds indicated that none of these unidentified indole derivatives was psilocybine[1] (R_f 0/0.27), tryptamine (R_f 0.78/0.67), or N,N-dimethyltryptamine[2] (R_f 0.93/0.69). Psilocybine was not detected in the carpophores of *Panaeolus campanulatus* in spite of the recent report by Heim and Wasson (16) of its isolation from *Panaeolus sphinctrinus*. This would indicate that *Panaeolus campanulatus* and *Panaeolus sphinctrinus* are not conspecific and argues for the designation of the former species as *Panaeolus linnaenus* Imai.

The absence of indole derivatives from the mycelial cultures of the organism is particularly interesting in view of the report of Singer and Smith (1) that *Panaeolus subalteatus* (*venenosus*) produces an active hallucinogenic principle, which is presumably an indole derivative, in its mycelium. This, too, is indicative of certain differences in the metabolism of various indole compounds in different species of the genus *Panaeolus*. Although some species may contain hallucinogenic agents, *Panaeolus campanulatus* does not, and it should cease to be designated as a mushroom capable of producing cerebral mycetism.

An extract of the fungus did exhibit parasympathomimetic activity which was muscarinic in nature. Chromatographic analysis revealed the presence of choline in the extract, but the occurrence of muscarine could not be confirmed. On the basis of the physiological tests, it is estimated that approximately 0.0013% of the fresh weight of the mushrooms corresponds to muscarine; slightly more than the 0.0003% of this compound shown to exist in *Amanita muscaria* (10). This low level of the material in the mushroom no doubt accounts for the inability to verify its presence by chromatographic analysis of a crude extract.

Since the fatal oral dose of muscarine in the adult human being varies between 0.3 and 0.5 Gm. (17), a quantity equivalent to more than 23 Kg. of fresh *Panaeolus campanulatus*, it must be concluded that the toxicity of this mushroom is extremely low. Although it probably will never serve as an important comestible because of its relatively small size and unpleasant taste, *Panaeolus campanulatus* should not be classified as a dangerously poisonous mushroom.

[1] Reference compound supplied through the courtesy of Dr. A. Hofmann, Sandoz A. G., Basel, Switzerland.

[2] Reference compound supplied through the courtesy of Dr. F. A. Hochstein, Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

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Colorimetric Determination of Dextrosulphenidol and Raceophenidol*

By EVAN W. McCHESNEY, JAMES M. SHEKOSKY, H. WILLIAM ECKERT, and RAYMOND F. KOSS

Dextrosulphenidol and raceophenidol may be degraded by alkaline hydrolysis, followed by periodate oxidation, to form *p*-methylsulfonylbenzaldehyde, which can be determined colorimetrically as the alkali salt of its *p*-nitrophenylhydrazone. Specific analytical procedures for the determination of dextrosulphenidol and raceophenidol in blood plasma or serum, urine, feces, tissues, eggs, and poultry feed, based on these reactions, are described.

THE SYNTHESIS of dextrosulphenidol (DES), or Win 5063-2, was first described by Cutler, Stenger, and Suter (1). Chemically the compound is D*d*-threo-2-dichloracetamido-1-(4-methylsulfonylphenyl)-1,3-propanediol. Bacteriologically it is a potent chemotherapeutic agent. Both it and the corresponding racemate[1] have been studied. The ultraviolet spectrum of DES is of such a nature (E = 13,700 at 223 mμ) (2) as to be of little value for analytical purposes, and assay has thus far been based on a tube dilution technique (3). Obviously, knowledge of the absorption, tissue distribution, and excretion of the compound could be enlarged greatly if a chemical method of analysis were available. It has been shown by Glazko, Dill, and Rebstock (4) that chloramphenicol may be degraded by alkaline hydrolysis, followed by periodate oxidation, to give *p*-nitrobenzaldehyde. DES, similarly treated, would yield *p*-methylsulfonylbenzaldehyde. If the latter compound could be isolated and determined chemically, the basis of an analytical method for DES, applicable to a wide variety of materials, would be at hand.

EXPERIMENTAL

Preparation of p-Methylsulfonylbenzaldehyde.—To 5 Gm. of DES[2] add 250 ml. of 0.1 *N* sodium hydroxide and heat on a boiling water bath until solution is complete, then continue heating for another thirty minutes. Cool to room temperature and adjust the pH electrometrically to 7.5 with phosphoric acid. Dissolve 5 Gm. of sodium metaperiodate in 150 ml. of water, and adjust the pH to 7.5. Combine the two solutions, then allow the oxidation to proceed for twenty minutes. During this time a white crystalline solid separates out; it is filtered off, washed with water, and recrystallized from ethyl acetate. Finally, the crystals are dried *in vacuo* over $CaCl_2$. Yield, 1.4 Gm.; m. p., 155°; m. p. in literature, 157° (5).

* Received July 22, 1959, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

[1] In the earlier literature DES was referred to as Thiocymetin, a name which is now reserved for the *dl* compound (generic name: raceophenidol). The methods of analysis described in this paper would be applicable to any optical isomers of the compound providing its specific distribution coefficient in the system: ethyl acetate/dilute phosphate is established. See footnote 3.

[2] Available from the Special Chemicals Division of the Winthrop Laboratories, 1450 Broadway, New York, N. Y.

Anal.—Calcd. for $C_4H_9SO_2$: C, 52.2; H, 4.35; S, 17.37. Found: C, 51.76; H, 4.51; S, 17.22.

Special Reagents.—

1. PNP solution. Eastman's *p*-nitrophenylhydrazine (100 mg.) is dissolved in 100 ml. of 5% perchloric acid; this reagent is prepared daily.
2. TEA solution. Eastman's tetraethylammonium hydroxide (10% solution) is diluted with 3 parts of absolute methanol.
3. Standard solution for colorimetry. *p*-Methylsulfonylbenzaldehyde is dissolved in chloroform (1 mg./ml.); a working standard is prepared by diluting this with $CHCl_3$ to a concentration of 3 mcg./ml.
4. Sodium metaperiodate, 0.5% aqueous solution.
5. Zinc acetate, 3% aqueous solution.
6. Sodium hydroxide, approximately *N* solution.
7. Sodium acid phosphate, 1.45 *M* solution.
8. Solvent mixture. One part methylene chloride diluted with two parts *n*-hexane. Neither of these solvents seems to require special purification, but if too large a reagent blank is obtained, the solvent mixture may be purified by successive washings with 1% bisulfite, 1 *N* NaOH, and water. It is then dried over Na_2SO_4 and filtered.
9. Phosphate buffer 0.4 *M*, pH 6.2.

Establishment of a Calibration Curve.—Measure into a series of 15-ml. glass-stoppered centrifuge tubes the following volumes of the dilute standard solution: 0, 1, 2, 3, 4, and 5 ml. Evaporate off the solvent, add 3 ml. of reagent 1, and heat the tubes in a boiling water bath for ten minutes. Cool to room temperature, add exactly 6 ml. of reagent 8 to each tube, and shake for one to two minutes. Draw off 5-ml. aliquots of the solvent phases and transfer them to a series of test tubes each of which contains exactly 2 ml. of reagent 2. Mix well; this converts the *p*-nitrophenylhydrazone to the quinoidal salt form (6). Aspirate off the upper phase and read the absorbance of the methanol phase in 2-ml. cuvettes, at 570 mμ. The calibration curve is linear over the specified range, and the concentration/absorbance relationship as determined in the Coleman Jr. spectrophotometer, may be expressed by the equation: 10 mcg. = 0.407 ± 0.008. (This amount of the aldehyde is equivalent to 19.1 mcg. of DES.) Once the calibration curve is definitely established, daily confirmation is unnecessary.

Alternate Method.—One may establish the calibration curve with DES itself as the starting material. Measure into a series of six 100-ml. beakers a methanolic solution of DES (1 ml. = 5 mcg.) in the amounts of 0–5 ml. Evaporate off the methanol, add 18 ml. of water and 2 ml. of reagent 6 to

each beaker, cover with watch glass, and digest on a boiling water bath for thirty minutes. Now add 3 ml. of reagent 7 to each sample, cool to room temperature, and transfer the solutions to 125-ml. separatory funnels. Shake each with 80 ml. of U. S. P. ether, followed by 80 ml. of U. S. P. chloroform. Run the aqueous phases back into the original beakers and digest on the steam bath for five to ten minutes to drive off the dissolved solvents. Transfer the solutions to 40-ml. centrifuge tubes, wash each beaker with 5 ml. of reagent 4, and add these washings to the solutions in the corresponding centrifuge tubes. Allow the oxidation reaction to proceed for twenty minutes, add 3 ml. of reagent 5 to each tube, and centrifuge briefly. Decant the supernatants into 50-ml. glass-stoppered graduated cylinders or centrifuge tubes, each of which contains 25 ml. of reagent grade chloroform. Shake for one to two minutes, allow the phases to separate (or centrifuge), aspirate off the aqueous phases, and transfer 20-ml. aliquots of the solvent phases to 50-ml. glass-stoppered centrifuge tubes. Evaporate off the solvent, add 3 ml. of reagent 1 to each, and proceed with the remaining steps as described above. For this procedure, the calibration factor would be 10 mcg. DES = 0.170 ± 0.003.

Principles of DES Analysis.—The periodate oxidation of extracts of biological materials might be expected to produce a number of aldehydes, principally formaldehyde. *p*-Methylsulfonylbenzaldehyde differs from most of these potentially interfering aldehydes in that it is nonvolatile. The objective of all of the fractionation procedures used in the analysis is to eliminate substances which might yield nonvolatile aldehydes, or which might prevent the oxidation reaction from going to completion by reducing excessive amounts of periodate. The essential steps in an analysis are: (*a*) aqueous homogenization (or simply, dilution) of the biological material; (*b*) partition between ethyl acetate[3] and water, to eliminate the predominantly water-soluble extractives; (*c*) partition between dilute methanol and hexane, to remove lipids; (*d*) alkaline hydrolysis of the residue from step *b* or *c*; (*e*) periodate oxidation; (*f*) chloroform extraction of the aromatic aldehyde; and (*g*) colorimetric determination of the aldehyde as the hydrazone. Since steps *d*–*g* are the same for all materials, they need not be outlined in detail for each, and they will be referred to below simply as the "standard operations." Steps *a*, *b*, and *c* are varied according to the nature of the material to be analyzed; in some instances steps *a* and *b* may be combined, or the order of steps *b* and *c* may be reversed, etc.

Procedures for Specific Materials.—*Urine Analysis* (*Total DES*).—Measure 1 ml. of urine into a test tube, add 2 ml. of reagent 6, stopper the tube with cotton, and immerse in a boiling water bath for one hour. (This hydrolysis should be sufficiently drastic to cleave a glucuronide conjugate of DES (4).) Cool to room temperature, dilute to about 10 ml., and transfer the solution to a 125-ml. separatory funnel with the aid of another 10 ml. of water. Add 3 ml. of reagent 7 and extract with 80 ml. of U. S. P. ether, followed by 80 ml. of U. S. P. chloroform. Run the aqueous phase into a 40-ml. centrifuge tube and wash the separatory funnel with about 2 ml. of water (into the centrifuge tube). Heat the tube in a boiling water bath for about five minutes; proceed as described under "Alternate Method" (i. e., add 5 ml. of reagent 4, allow the oxidation reaction to proceed for twenty minutes, add 3 ml. of reagent 5, mix,[4] centrifuge briefly, etc.). A convenient aliquot of chloroform to be evaporated for the PNP reaction is 2 ml.; if this proves to be too large or too small (absorbance more than 0.9 or less than 0.15) the PNP reaction is repeated on the indicated smaller or larger aliquot. The recovery of DES from urine in the range 10–160 mcg./ml. is 95 ±7%, and the blank on human urine is of the order of 10 mcg./ml.

Urine Analysis (*Unchanged DES*).—Measure 5 ml. of urine into a 250-ml. separatory funnel, add 15 ml. of absolute methanol, and extract with 80 ml. of *n*-hexane which is discarded. Evaporate the methanol phase to dryness in a 150-ml. beaker, take up the residue in 25 ml. of reagent 9, and return the solution to the same separatory funnel. Extract with 125 ml. of ethyl acetate, discard the aqueous phase, and extract the solvent with another 10 ml. of reagent 9. Evaporate the solvent to dryness in a 250-ml. beaker, add to the residue 18 ml. of water, 2 ml. of reagent 6, and proceed with the hydrolysis, etc., as described under "Alternate Method" (standard operations). It remains for this, and all other analyses, to select a chloroform aliquot appropriate to the amount of DES believed to be present in the original sample. In this particular case, with the total aromatic aldehyde contained in 25 ml. of chloroform and each ml. of chloroform equivalent to 0.2 ml. of urine, an aliquot of 0.5 to 1 ml. may be sufficient (or even excessive) for the PNP reaction. A reagent blank based on 3 ml. of reagent 1 and 6 ml. of reagent 8 should be run simultaneously with the unknowns. This blank should give a net absorbance (against reagent 2) of about 0.04. It is also necessary to run comparable standards through the entire procedure, since some of the DES is fractionated out in the ethyl acetate/phosphate partition.[3] This partition factor may be determined as follows: Measure into a 250-ml. separatory funnel 1 ml. of a methanolic solution of DES (1 ml. = 1 mg.), add 14 ml. of methanol, 5 ml. of water, and proceed with the hexane extraction, etc., as described for the urine analysis. Of the final chloroform solution of the aromatic aldehyde (25 ml.), 0.5 and 1 ml. will give the absorbances equivalent to initial concentrations of 20 and 40 mcg. of DES. A solvent blank is usually unnecessary, since it would be negligibly different from the reagent blank described above in this paragraph. The calculation of the result is very straightforward. Except for the fact that about 10% of the DES remains in the aqueous phase in the ethyl acetate/phosphate partition, the recovery from urine is 100%, and the precision of the analysis is equal to that of the calibration curve itself.

[3] Ethyl acetate is by no means an ideal solvent for this application, but it is the best which has been found. The partition coefficient of DES and racemycin in the system ethyl acetate/0.4 *M* phosphate is only 0.9–1.0, and must be established for each specific set of conditions. This amounts, in essence, to carrying standards through all of the procedural steps in about the amounts expected to be present in the material being analyzed.

[4] The purpose of adding the zinc acetate is to precipitate the excess periodate. If no precipitate forms, the amount of periodate added was insufficient, and that sample must be repeated with a larger amount of the oxidant.

Serum Analysis (for Unchanged DES).—Measure into a 100-ml. glass-stoppered centrifuge tube 3 ml. of serum; if less is available, add water to make up the difference. Add 1 ml. of reagent 7, then 75 ml. of reagent grade ethyl acetate, and shake for fifteen minutes. Centrifuge briefly; transfer 70 ml. of the clear solvent phase to a 150-ml. beaker for evaporation to dryness. Take up the residue in 30 ml. of 75% methanol and extract with 60 ml. of *n*-hexane. Return the methanol phase to the same beaker, discard the hexane, and wash the separatory funnel with 5 ml. of methanol. Evaporate the combined methanol extract and washing to dryness; the separatory funnels may be used again in the subsequent steps. Now proceed with the standard operations of alkaline hydrolysis, etc. The final chloroform extraction of the aromatic aldehyde is made with 25 ml., and an aliquot of 22 ml. is evaporated in a 50-ml. glass-stoppered centrifuge tube for the PNP reaction. A reagent blank and DES standards (10 and 20 mcg.) should be carried through the entire procedure simultaneously (substituting water for serum), and the calculations should be based on these standards. In our hands, a reagent blank gives an absorbance of 0.045 ± 0.005, and for each increment of 10 mcg. of DES in the original sample the increment of absorbance is 0.127 ± 0.005. The smallest amount of DES which can be satisfactorily determined is 2 mcg., the recoveries of DES from serum at various concentration levels are given in Table I.

Tissue Analysis.—The determination of DES in tissues may be based on the general principles of the Levine and Fishbach procedure for chloramphenicol (7). Two essentially different analytical problems may be distinguished: (*a*) the determination of very small antibiotic residues (<1 p. p. m.) in large tissue samples and (*b*) the determination of more normal antibiotic concentrations in metabolism experiments on small laboratory animals. The specific procedures follow:

(*a*) Homogenize 25 Gm. of tissue in a Waring Blendor with 85 ml. of water, add 15 ml. of reagent 7, and transfer the homogenate to a 1-L. glass-stoppered bottle. Add 500 ml. of U. S. P. ethyl acetate, and shake for fifteen minutes. Allow the phases to separate, then decant as much of the solvent as possible into a 500-ml. separatory funnel in which it is washed with 40 ml. of reagent 9. Draw off the aqueous phase, add 5 Gm. of filter cel to the solvent, shake well, and filter into a graduated cylinder. Transfer 425 ml. of the clear solvent to a 600-ml. beaker and evaporate it to dryness on the steam bath. Take up the residue in 75 ml. of 75% methanol and extract twice with 150 ml. of *n*-hexane. (If the methanol phase is not clear and colorless, a third hexane extraction is indicated.) The methanol phase and methanol washings of the separatory funnel are now evaporated to dryness (or nearly so) in a clean 250-ml. beaker. Water is added to bring the volume up to 18 ml., 2 ml. of reagent 6 is added, and the "standard operations" are performed. The final extraction of the aromatic aldehyde is made with 25 ml. of chloroform, of which 22 ml. is evaporated for the PNP reaction. Standards of 0–20 mcg. DES in 85 ml. of water plus 15 ml. of reagent 7 should be carried through the entire procedure to determine the colorimetric factor applicable to this specific analysis.

This procedure has been tested on a series of homogenates of chicken muscle and beef liver (25 Gm. in each homogenate), adding the compound by dissolving it in the water in which the tissue was homogenized. Twenty-seven muscle homogenates and seven liver homogenates were prepared; of these, twelve muscle and three liver homogenates were retained to establish the tissue blanks, and DES was added in various amounts to the other samples. The results of these experiments are presented in Table II.

(*b*) Homogenize 2 Gm. (or less, if the organ does not weigh that much) with 8 ml. of water and add 2 ml. of reagent 7. Shake the homogenate in a glass-stoppered centrifuge tube with 40 ml. of ethyl acetate for fifteen minutes, clarify by centrifugation, and evaporate 35-ml. aliquots of the solvent in 100-ml. beakers. Take up each residue in 55 ml. of 75% methanol and extract with 165 ml. of *n*-hexane, in a 250-ml. separatory funnel. The methanol phase and methanol washings of the separatory funnel are returned to the same beaker and are evaporated to dryness, after which the "standard operations" are performed on the residue. DES standards in the range 0–50 mcg. are carried through the same processing steps as the tissue, maintaining identical volume relationships. Both muscle and liver have been analyzed in this manner, with the results presented in Table III. The DES to be recovered was added as described under (a).

Analysis of Feces.—Transfer 3 Gm. of dry, finely ground feces (or the entire sample, if it weighs less than 3 Gm.) to an 8-ounce glass-stoppered bottle, add 20 ml. of water, 5 ml. of reagent 7, and 125 ml. of ethyl acetate. Shake for twenty minutes, allow the phases to separate, and transfer 100 ml. of the solvent to a 250-ml. beaker in which it is evaporated to dryness. Take up the residue in 75 ml. of 75% methanol and extract it thoroughly with 150 ml. of hexane. Return the methanol phase (with methanol washings of the separatory funnel) to the original beaker and evaporate to dryness. Proceed with the "standard operations" of hydrolysis, etc. The final chloroform extraction of the aromatic aldehyde is made with 25 ml., of which a 5-ml. aliquot is evaporated in a 15-ml. glass-stoppered centrifuge tube for the PNP reaction. If this aliquot proves to be too large or too small, the PNP reaction is repeated on the indicated aliquot. Standard amounts of DES (500 and 1,000 mcg.) are carried through the same processing steps and the results of the analyses are evaluated from them. The reagent blank may be be based on reagents 1 and 8 only (3 and 6 ml., respectively).

DES has been added to 3-Gm. samples of dry rat feces and processed as above. The blank value obtained was 8 mcg./Gm., and the recovery, in the range of 500–1,500 mcg. added (per 3 Gm.), was 90–100%.

Determination of DES in Eggs.—The edible part of the egg is homogenized with 400 ml. of acetone, the homogenate digested on the steam bath for fifteen minutes; cooled to room temperature, and filtered. The filtrate and acetone washings of the insoluble residue are evaporated and the residue from this evaporation is taken up in 75 ml. of 75% methanol. The solution is extracted twice with 300 ml. of *n*-hexane which is discarded. The methanol phase is clarified by centrifugation and evaporated to dryness. Another 75 ml. of absolute methanol is used

TABLE I.—RECOVERY OF DEXTROSULPHENIDOL FROM 3-ML. SAMPLES OF HORSE SERUM

mcg. Added[a]	mcg. Found	mcg. Recovered	Analytical Error mcg./ml.	Analytical Error %
0	0.93	...	...	...
2	2.87	1.94	−0.02	− 3
4	4.65	3.72	−0.09	− 7
8	7.37	6.44	−0.52	−19
12	10.00	9.07	−0.98	−24
16	15.10	14.17	−0.61	−11
20	19.75	18.82	−0.39	− 6
30	29.10	28.17	−0.61	− 6
		Mean ± S. D.	−0.46 ± 0.33	−11 ± 8
0[b]	0.46 ± 0.22[b]	...	...	...
3[c]	3.47 ± 0.54[c]	3.01 ± 0.41[d]	+0.01 ± 0.14[d]	0 ± 14[d]

[a] One sample analyzed at each level.
[b] Mean ± S. D. of 10 samples.
[c] Mean ± S. D. of 12 samples.
[d] Deviation calculated on the basis of the principle of pooled variance.

TABLE II.—RECOVERY OF DEXTROSULPHENIDOL FROM 25-GM. HOMOGENATES OF CHICKEN MUSCLE AND BEEF LIVER

No. of Samples	mcg. Added	mcg. Found	Net Recovery mcg.	Net Recovery %
12[a]	0	1.25 ± 0.32[c]	...	...
12[a]	5	6.75 ± 0.90[c]	5.50 ± 0.70[c]	110 ± 14[c]
1[a]	10	13.25	12.0	120
1[a]	20	18.50	17.25	86
1[a]	40	39.70	38.45	96
3[b]	0	2.5 ± 2.2[c]	...	...
1[b]	5	5.5	3.0	60
1[b]	10	12.5	10.0	100
1[b]	20	19.8	17.3	87
1[b]	40	43.2	40.7	102

[a] Chicken muscle. [b] Beef liver. [c] Mean ± S. D.

TABLE III.—RECOVERY OF DEXTROSULPHENIDOL FROM 2-GM. TISSUE HOMOGENATES

mcg. Added	mcg. Found Muscle	mcg. Found Liver	Net Recovery Muscle mcg.	Net Recovery Muscle %[a]	Net Recovery Liver mcg.	Net Recovery Liver %[a]
0	0.90, 0.20	−0.40, −0.40	...	...	...	...
5.0	5.4	3.6	4.9	98	4.0	80
10.0	10.4	9.0	9.9	99	9.4	94
20.0	19.0	14.4	18.5	93	14.8	74
40.0	43.4	17.4	42.9	106	17.8	89
			Mean recovery	99		84

[a] Of amounts added.

to wash out the separatory funnel and centrifuge tube to minimize loss on the glassware. The combined methanolic extracts are evaporated to dryness and the residue is further processed in the usual way: it is partitioned between 25 ml. of reagent 9 and 125 ml. of ethyl acetate; the latter is washed with another 10 ml. of reagent 9, and is then filtered through Na_2SO_4 or a double filter paper into a 250-ml. beaker, in which it is evaporated to dryness. The "standard operations" are performed on the residue. A convenient volume of chloroform for extraction of the aromatic aldehyde is 25 ml., of which 22 ml. are evaporated for the reaction with PNP. Standards containing 0, 10, and 20 mcg. of DES in 400 ml. of acetone are evaporated and processed as described for the eggs to establish the colorimetric factor applicable to this analysis.

Eggs usually give a somewhat "off" (brown) color at the last step of the analysis. This "off" color can be compensated for, to a certain extent, by making absorbance readings at both 570 and 700 mμ and taking the net absorbance (570–700) as the true value. If this is done, the standards are also read in the same way. Practically no sensitivity is sacrificed thereby, but the egg blank is significantly reduced. The recovery of DES from acetone homogenates of eggs is shown in Table IV.

Determination of Raceophenidol in Poultry Feed.—To 5 Gm. of feed in a 100-ml. glass-stoppered graduated cylinder add 100 ml of reagent grade acetone, shake for five to ten minutes, and filter. Transfer 75 ml. of the clear filtrate to a 250-ml. Erlenmeyer flask and evaporate it to dryness. Add to the residue 25 ml. of reagent 9 and exactly 125 ml. of

TABLE IV.—RECOVERY OF DEXTROSULPHENIDOL FROM ACETONE HOMOGENATES OF EGGS

No. of Eggs	mcg. Added[a]	mcg. Found[a]	Net Recovery mcg.	Net Recovery %
3	0	0.9 ± 1.1[b]	...	...
1	5	3.9	3.0	60
1	10	8.2	7.3	73
1	20	17.3	16.4	82
1	40	32.8	31.9	79

[a] Per egg. [b] Mean ± S. D.

ethyl acetate; shake mechanically for five minutes. Aspirate off the aqueous phase, add about 5 Gm. of filter cel to the solvent phase, and filter the latter into a 100-ml. graduated cylinder or volumetric flask. When 100 ml. of filtrate has been collected transfer it to a 250-ml. beaker and evaporate to dryness.

Now proceed with the standard operations of hydrolysis, etc., finally extracting the aromatic aldehyde into 25 ml. of chloroform. Of this, transfer 5 ml. to a 40-ml. glass-stoppered centrifuge tube and proceed with the *p*-nitrophenylhydrazine reaction in the usual way. Read the absorbances at 570 and 700 mμ, taking the net value (570–700) as the true reading. The results are evaluated against raceophenidol standards (0, 100, and 200 mcg. in 75 ml. of acetone) carried through the entire procedure. The sample finally analyzed represents 0.6 Gm. of feed.

Six samples of feed were analyzed as described above; two were blanks and four were mixed to contain 25 Gm./ton of raceophenidol. The results were as follows: blank feed, −0.25, −0.25 Gm./ton; medicated feed, 22.0, 23.5, 24.9, 23.5 Gm./ton; mean net recovery, 23.75 ± 1.2 Gm./ton, or 95 ± 5% of claim.

It may be noted that the readings on the blank feed were less than the reagent blank, therefore, the negative values for these samples.

CONCLUSIONS

Dextrosulphenidol and raceophenidol may be determined colorimetrically in a wide variety of materials as the *p*-nitrophenylhydrazone of *p*-methylsulfonylbenzaldehyde. The recovery from the materials studied ranges from 70 to 100%; blank values on such materials as tissues and eggs do not usually exceed 0.2 p. p. m. The least amount of these compounds which can be determined satisfactorily is 1 mcg. at the last step of the analysis. This requires the presence of at least 2–3 mcg. in the initial sample.

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Some Aspects of Chemical and Pharmacological Studies of *Acorus calamus* Linn.*

By B. C. BOSE, R. VIJAYVARGIYA, A. Q. SAIFI, and S. K. SHARMA

In the present investigation, chemical as well as pharmacological studies of *Acorus calamus Linn.* an indigenous medicinal plant used in asthma, have been carried out. The chemical analysis revealed the presence of reducing sugars, tannins, resins, sterol, 1.5 per cent of essential oil, 0.4 per cent of potassium, and 0.6 per cent calcium. The essential oil and alcoholic and aqueous extracts showed depressant action on normotensive mongrel dogs. They also inhibited the rhythmicity of frog and dog heart and relaxed the tone of isolated intestine, uterus, and bronchi and antagonized acetylcholine and histamine-induced spasm. The former action was not modified by atropinization and the latter by previous treatment with antihistaminic drugs. Among the various fractions, essential oil was found to be the most active and appeared to be the active principle of the plant. The hypotensive property of the alcoholic extract, as reported by Agarwal, also seemed to be due to this fraction. The bronchodilator effect of the essential oil, as observed in lung perfusion and isolated tracheal chain experiments, was much weaker than that of adrenaline.

Acorus calamus Linn., a semiaquatic perennial plant, belonging to the family of *Araceae*, is widely grown in India and known as "Buch." The plant is popularly used in indigenous medicine for asthmatic conditions. Recently, Chopra (1), Agarwal (2), and Dandiya (3) have described the hypotensive and sedative properties of the alcoholic extract, as well as the essential oil obtained from the plant. Volatile oils, collected from *A. calamus* of Indian and European origin, have been reported to possess differences in chemical and pharmacological properties.

In view of the above observations, it was considered advisable to carry out further chemical and pharmacological studies on this plant, with particular reference to its bronchodilator effect, for which, as stated above, the plant is being empirically used in indigenous medicine.

METHODS AND RESULTS

In this investigation, chemical and pharmacological studies on *Acorus calamus* of Indian origin, col-

* Received June 15, 1959, from the Department of Pharmacology, M. G. M. Medical College, Indore, India.

lected from Dehradun, have been carried out.

The rhizomes and roots, powdered and stored in airtight containers, were subjected to successive extraction with the following solvents and the percentage residues determined; petroleum ether, 5.66; benzene, 1.33; ether, 0.38; chloroform, 1.20; alcohol, 2.21; and water, 5.74.

The chemical analysis of the above extracts revealed the presence of reducing sugars, tannins, resins, and sterol bodies. The drug was also found to contain 1.5% volatile oil as estimated by essential oil-determination apparatus, resins 2.5% by A. O. A. C. method, and tannins 1.5% by potassium permanganate titration method.

The drug yielded 4.8% of total ash, 0.05% acid-insoluble ash, and 0.46% water-soluble ash, which on further analysis, showed the presence of potassium, sodium, calcium, iron, and aluminum.

Potassium, sodium, and calcium contents in aqueous and alcoholic extracts were analyzed in B. Lange flame photometer, model 5, which revealed the following figures: potassium 0.499 and 0.423%; sodium 0.289 and 0.40%, and calcium 0.60 and 0.60% in aqueous and alcoholic extracts, respectively.

The detailed pharmacological investigations were then carried out with the aqueous and alcoholic extracts and essential oil fraction. On preliminary analysis, petroleum ether, benzene, ether, and chloroform extracts did not show any significant pharmacological responses.

Blood Pressure.—It was studied on 10 mongrel dogs, weighing 9–15 Kg. and anesthetised with Dial. The essential oil, in a dose range of 0.01–0.024 ml./Kg., produced a marked fall in blood pressure, as recorded in Table I and Fig. 1.

Alcoholic and aqueous extracts also produced a fall in blood pressure but not to the same extent as in the case of the volatile oil. Antihistaminic and atropine group of drugs did not block the hypotensive action of the drug.

Heart in Situ.—On frog's heart *in situ*, essential oil produced the depression both in rate and amplitude, in a dose range of 1:10,000 to 1:100, as shown in Fig. 2. Aqueous and alcoholic extracts also caused inhibition of the heart, though to a lesser extent. In dog heart, depression was observed both on auricles and ventricles *in situ*.

TABLE I.—EFFECTS OF AQUEOUS AND ALCOHOLIC EXTRACTS AND ESSENTIAL OIL FRACTION OF *A. calamus* ON BLOOD PRESSURE OF DOG

Extract and Dose	Average Initial Blood Pressure, mm. Hg	Average Blood Pressure after Drug, mm. Hg	Average Fall in Blood Pressure, %
Aqueous, Gm./Kg.			
0.020	122/102	102.4/85.6	16.60
0.040	126/106	99.5/85.8	21.00
0.060	125/105	93.7/73.8	25.00
Alcoholic, Gm./Kg.			
0.020	126/106	99.5/85.6	21.00
0.040	125/105	93.1/83.2	25.50
0.060	122/102	85.4/71.4	30.00
Essential oil, ml./Kg.			
0.012	110.5/105.5	60.0/57.3	45.7
0.018	105.0/100.0	50.6/48.2	51.8
0.024	100.0/ 90.0	59.7/35.7	60.3

Isolated Intestine.—Aqueous and alcoholic extracts, as well as essential oil, produced a depressant effect on the tone and rhythmicity of isolated intestine of guinea pigs and rabbits. Essential oil, in a dose range of 0.2×10^{-5} to 10^{-3}, was found to be the most potent as shown in Fig. 3. The spasmogenic effect of acetylcholine, histamine, and barium chloride was also antagonized.

Isolated Uterus.—The action of essential oil was estimated on rat uterus. In a concentration of 1:500, it depressed the tone and rhythmicity and

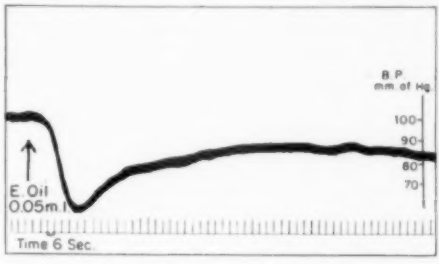

Fig. 1.—The effect of essential oil on blood pressure of mongrel dogs anesthetized with Dial.

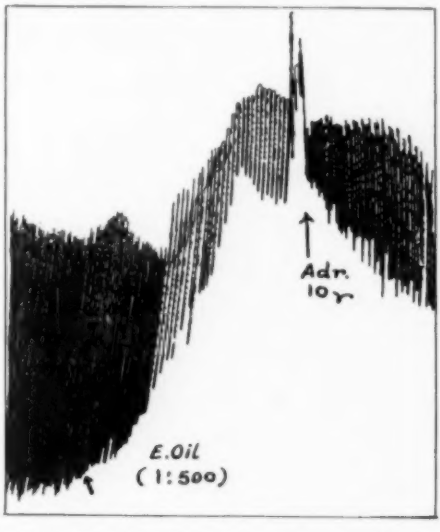

Fig. 2—The effect of essential oil on frog heart *in situ*.

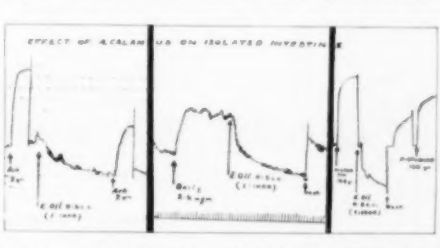

Fig. 3.—The effects of essential oil and aqueous and alcoholic extracts on isolated intestine.

also antagonized the acetylcholine-induced contractions. The findings are shown in Fig. 4.

Respiration.—The effect was studied on 10 Dial-anesthetized mongrel dogs, weighing 9–11 Kg. Alcoholic and aqueous extracts, in a dose range of 0.06 to 1.10 Gm./Kg., produced a brief apnea, followed by a slight stimulation. On the other hand, essential oil, in a concentration of 0.012 to 0.018 ml./Kg., depressed the respiration, and in higher doses of 0.024 ml./Kg., it produced Cheyne-Stoke respiration as shown in Fig. 5. Atropine and antihistaminics did not modify this response.

Isolated Tracheal Chain.—Guinea pigs, weighing between 350–500 Gm., were stunned and exsanguinated by incision of carotids. After separating from deeper structures, trachea was removed in oxygenated modified Ringer. Six to eight units (two rings in each unit) were tied from end to end so that the tracheal muscles were aligned. The cartilage was incised in the center and hung in an isolated organ bath. Effects of essential oil and alcoholic and aqueous extracts were studied against the contractions induced by acetylcholine (10^{-6} to 2×10^{-5}) and histamine (10^{-6} to 0.2×10^{-5}). Drugs were allowed to act for two and one-half minutes before adding the spasmogenic agents and the percentage inhibition calculated as shown in Table II and Fig. 6.

From Table II it will be seen that aqueous and alcoholic extracts in a dose of 15 mg./ml. caused inhibition of acetylcholine contractions by about 68% while histamine contractions were reduced by about 50%. Essential oil in a concentration of 1:5,000 antagonized acetylcholine-induced contractions by about 80% as against 55% reduction of histamine contraction.

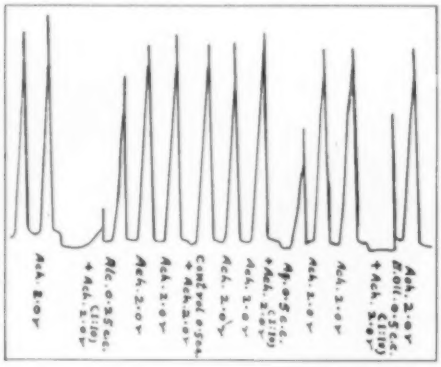

Fig. 4.—The effect of essential oil on isolated rat uterus.

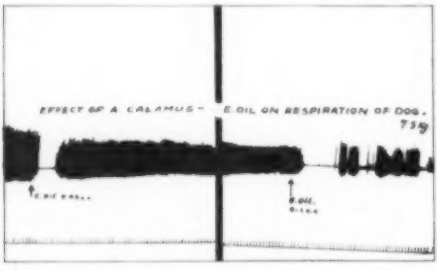

Fig. 5.—The effect of essential oil on respiration in Dial-anesthetized dog.

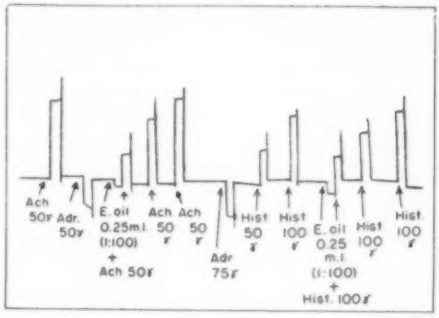

Fig. 6.—The effect of essential oil on guinea-pig tracheal chain.

TABLE II.—EFFECTS OF AQUEOUS AND ALCOHOLIC EXTRACTS AND ESSENTIAL OIL FRACTIONS OF *A. calamus* ON ACETYLCHOLINE AND HISTAMINE-INDUCED CONTRACTIONS ON ISOLATED TRACHEAL CHAIN OF GUINEA PIG

Extract	Concentration	Percentage Inhibition Acetylcholine 10^{-6} to 0.2×10^{-5}	Percentage Inhibition Histamine 0.2×10^{-6} to 10^{-5}
Aqueous	5.0 mg./ml.	38.00	25.60
	10.0 mg./ml.	54.38	42.80
	15.0 mg./ml.	68.80	55.24
Alcoholic	2.5 mg./ml.	32.82	23.60
	5.0 mg./ml.	49.35	38.18
	10.0 mg./ml.	67.72	50.12
Essential oil	1:15,000	25.10	20.80
	1:10,000	50.31	39.20
	1: 5,000	80.40	55.90

Lung Perfusion.—The technique of Tainter (4) was followed. In a dose of 150 mg., alcoholic and aqueous extracts produced about 33% of bronchodilator action. Essential oil in a dose of 0.1 ml. caused 32% dilatation, as measured by perfusate. Antagonism to acetylcholine constriction by alcoholic and aqueous extracts was observed to the extent of 65.7% and 60.1%, respectively. Histamine constrictions were inhibited by 56.9% and 54.5%, respectively, by the above doses. Essential oil antagonized acetylcholine and histamine bronchoconstrictions by 65 and 58.9%, respectively, in a concentration of 0.01 ml. On comparing this bronchodilator action with epinephrine, essential oil was found to be 50 times less active than the former.

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The Physics of Tablet Compression X*

Mechanism of Action and Evaluation of Tablet Lubricants

By W. A. STRICKLAND, Jr., TAKERU HIGUCHI, and L. W. BUSSE

A study of theoretical aspects of tablet lubrication has been carried out in an attempt to elucidate the mechanism of lubricant action and the relationships that exist between chemical structure and lubricating properties. The results of this study indicate that the mechanism of lubricant action, as described for other systems, may be applicable to tablet lubrication. Thus, lubricants may generally be divided into two groups; the first known as fluid- (or hydrodynamic) type which is exemplified by hydrocarbons and the second, termed boundary-type lubricants such as the metallic stearate salts. There appears to be correlation between chemical structure and lubricant efficiency in the tablet-die wall system. The effectiveness of fluid-type lubricants appears to be related to the viscosity of the material used. In this system paraffin was more effective than liquid mineral oil. The evaluation of other classes of compounds revealed that metallic salts of fatty acids were generally the best lubricants while fatty acids, fatty alcohols, alkyl sulfonates, fatty amides, and certain polyvinyl and polyethylene glycol derivatives exhibited good lubricating properties. However, silicone resins, inorganic salts, proteins, carbohydrates, and many metal oxides displayed no lubricating properties. Included in the paper are the results of a survey in which 70 materials were evaluated as tablet lubricants.

ALTHOUGH the mechanism of lubricant action has been extensively studied for systems involving metal-metal and crystal-crystal friction (6, 7), there appears to have been no previous attempt made to correlate these findings with the behavior of lubricants in the tablet compression process. The purpose of this paper is (*a*) to present certain aspects of friction and lubrication theory as it may apply to tablet lubrication; (*b*) to report results of a study designed to show that, in general, lubrication theory which has been previously described for other systems is applicable to the tablet-die wall system; and (*c*) to show that the relative values of various materials as tablet lubricants can be correlated in part with their chemical and physical nature.

As a part of this study a survey was made of various substances which may be used as lubricants. Results of the experimental evaluation of 70 different materials are presented in this paper.

EXPERIMENTAL

The experimental procedures, which were similar to those described in previous reports (1, 2), employed the instrumented tablet machine equipped with $^3/_8$ inch flat-faced punch and die sets. Tablets were compressed under a total force of 800 to 1,200 Kg. (1,100 to 1,700 Kg. per sq. cm.). Test granulations were starch paste granulations of sulfathiazole and sodium bicarbonate seived to collect 10–20-mesh granules. Lubricants were added as 100-mesh or finer powders unless otherwise noted. Liquid lubricants and solutions were applied to tumbling granulations as ethereal sprays.

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THEORY OF LUBRICATION AND TABLET COMPRESSION

During compression of a tablet on a single punch machine the resistance to the downward stroke of the upper punch is due to packing of the granulation and the frictional retarding effect at the granule-die wall interface. After compression the resistance to tablet ejection is also due to the tablet-die wall friction.

The tablet compression process generally involves friction between two polar surfaces of hard texture. One of the materials concerned in the operation is usually steel, which according to published reports (4, 5) is coated with an oxide film approximately 100 Å. thick. The other materials in this consideration of friction are the granulations and subsequently formed tablets. The granulations, in absence of lubricants, have surfaces consisting of starch and drug on which is adsorbed atmospheric oxygen, nitrogen, and water vapor.

The problem of the tablet maker is to minimize, by addition of lubricants, these frictional forces between the die wall and granulation as the tablet is formed and ejected. Lubrication in all cases consists of interposing small amounts of a suitable material between two rubbing surfaces; the purpose being to reduce the friction arising at the interface. The action of lubricants is generally considered to occur by two mechanisms (6, 7). The first is termed fluid (or hydrodynamic) lubrication because the two surfaces are separated by a finite and continuous layer of fluid lubricant, which under ideal conditions, prevents wearing of the solid surfaces. Hydrocarbons, such as mineral oil, are examples of fluid-type lubricants.

Boundary lubrication, the second mechanism, results from the adherence of polar portions of molecules with long carbon chains to the metal surfaces.

Magnesium stearate and stearic acid are examples of boundary lubricants. Evidence is presented to show that both of these processes are operative in tablet lubrication.

Observations on Fluid-Type Lubricants.—The study of fluid-type lubrication in tablet compression was carried out by coating the die wall with hydrocarbons of six different viscosities. They were white ceresin, white paraffin, paraffin and white petrolatum (equal parts w/w), white petrolatum, white petrolatum and mineral oil (equal parts w/w), and mineral oil, heavy. In order to cover the die wall with a relatively smooth coat of sufficient thickness to assure maximum lubrication, those hydrocarbons which were solid or semisolid at room temperature were melted and the die was warmed above the melting point of the material. After the die had been coated by means of a cotton swab dipped in melted hydrocarbon, it was allowed to cool to room temperature, placed in the tablet machine, and filled with the usual weight of unlubricated sodium bicarbonate test granulation.

Six or more tablets were subsequently compressed using unlubricated granulation without further treating the die. This was intended to demonstrate the tenacity with which lubricant can cling to die walls. The experimental values obtained were somewhat irregular due to the unavoidable variations in the amount of hydrocarbon applied to the die wall and the tendency of the lower punch to spread lubricant over the die wall in its cycle; however, these variables did not appear to affect the overall results appreciably.

The information derived from this procedure, which is summarized in Table I, indicated that the fluid mechanism of lubrication was operative in this system as in other systems previously reported. It appeared that the forces employed in these tablet compressions were of such magnitude that the viscosity of a liquid material was inadequate (Fig. 1), and only the waxy solids, paraffin and ceresin, had sufficient viscosity to prevent breakdown of the fluid layer and subsequent seizure of the two surfaces.

Upon compression of test granulations lubricated with mineral oil a peculiar behavior was encountered which appeared to be a manifestation of the stick-slip type of frictional phenomenon similar to that described in other systems (6). During compression, the two markers of the oscillograph used to measure

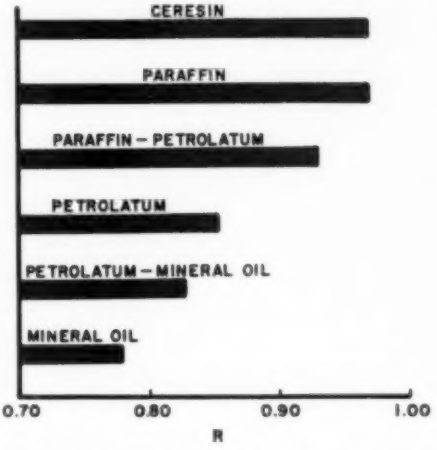

Fig. 1.—Comparison of maximum lubrication attainable with various hydrocarbon lubricants illustrating the dependence of lubricating properties on viscosity.

punch forces did not describe a smooth cycle but instead jerked up to a stop, then up again to yield a jagged, saw-toothed type of punch force record. The tablet machine emitted an audible groaning noise.

Figure 2 is a record of the punch forces *versus* time plot from the instrumented tablet machine when compressing sodium bicarbonate test granulation in a die coated with a thin film of mineral oil. This illustrates the type record obtained from compressions exhibiting stick-slip friction. It should be understood that the presence of this frictional phenomenon is relatively independent of the overall frictional resistance because it was encountered, to a lesser extent, with white petrolatum coated on the die wall even though there was a visible film of hydrocarbon and the R values at maximum punch forces were as high as 0.88.

This type frictional phenomenon was not found with any lubricant material which might be considered as a boundary-type lubricant other than lauryl alcohol. The alcohols, because of their weak polarity as compared to the fatty acids, probably do

TABLE I.—RESULTS OF COATING THE DIE WALL WITH HYDROCARBONS OF DIFFERENT VISCOSITIES FOLLOWED BY COMPRESSION OF SIX SUCCESSIVE TABLETS OF UNLUBRICATED SODIUM BICARBONATE TEST GRANULATION WITHOUT FURTHER TREATING THE DIE[a]

Material Used to Coat Die Wall		Tablet Number 1	2	3	4	5	6
Ceresin (white)	R Value	0.97	0.90	0.84	0.76	0.70	0.66
	Ejection force, Kg.	8	20	40	85	123	135
Paraffin	R Value	0.97	0.87	0.81	0.75	0.74	0.69
	Ejection force, Kg.	10	42	60	72	97	115
Paraffin-petrolatum (equal parts w/w)	R Value	0.93	0.81	0.74	0.73	0.71	0.71
	Ejection force, Kg.	36	65	82	100	120	115
White petrolatum U. S. P.	R Value	0.86	0.79	0.76	0.77	0.74	0.72
	Ejection force, Kg.	40	69	75	80	98	92
Petrolatum-mineral oil (equal parts w/w)	R Value	0.83	0.77	0.71	0.72	0.74	0.70
	Ejection force, Kg.	47	70	110	90	105	115
Mineral oil, heavy U. S. P.	R Value	0.78	0.77	0.82	0.75	0.77	0.75
	Ejection force, Kg.	46	60	80	80	80	90

[a] Tablet No. 1 represents the maximum lubrication attainable. R is the ratio of lower to upper punch force at the maximum force of compression.

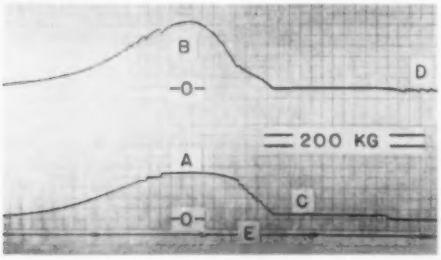

Fig. 2.—A photograph of the punch forces *versus* time record obtained from the Sanborn Oscillograph during compression of a sulfathiazole tablet when the die wall was coated with a thin film of mineral oil. This irregular tracing results from stick-slip type friction. *A*, The lower punch force record; *B*, the upper punch force record; *C*, ejection force record, the portion of the lower punch force record which denotes the force necessary to eject the formed tablet from the die; *D*, record of force exerted upon the upper yoke of the machine as it assumes the burden of ejecting the tablet, it could not be calibrated and was not used in collecting data; –0–, the base calibration line at which the punch forces are zero, each major division above the base line represents 200 Kg. force; and *E*, line recorded by the time marker, each pip on this line denotes one second of time.

not orient sufficiently strong to be considered as examples of boundary lubricants (7).

Observations on Boundary-Type Lubricants.—The adherence of a boundary lubricant to the die wall should be somewhat greater than that of a fluid lubricant. This may be expected for two reasons; first, the surface chemistry of lubricants (4, 5, 8) indicate that the polar end of the boundary lubricant molecule should adhere more tenaciously to the oxide-metal surface than the nonpolar fluid-type lubricant; and secondly, smaller quantities of boundary lubricants were required to give adequate lubrication. This second point was repeatedly supported by this work.

Maximum lubrication of the sodium bicarbonate test granulation was obtained by first lubricating a sample of the granulation with 2% magnesium stearate, then coating the inside die wall with the same material. This was accomplished by shaking an excess (20–40 mg.) of lubricant in the die cavity until the inside was completely covered, the excess tapped out, and the die placed in the tablet machine. Stearic acid (100-mesh) was also used in the same manner. One initial tablet of sodium bicarbonate test granulation containing 2% lubricant was compressed followed by seven more tablets of unlubricated granulation without further disturbing the die.

The results of this procedure cannot be compared quantitatively with the data of the similar procedure using hydrocarbon lubricants because of the difference in amount of lubricant that adhered to the treated die prior to compressions. The weight of hydrocarbon lubricant that was coated on the die wall was 25 to 50 mg. while the weight of magnesium stearate was three to eight mg. However, this large discrepancy does not reduce the value of comparison between the two types of lubricants, rather, it makes more evident the apparent superiority of the boundary-type lubricants. The pertinent information obtained in this study is shown in Table II.

CHEMICAL STRUCTURE AND LUBRICATING PROPERTIES

Existence of some relationship between lubricating properties of various materials and their chemical structure is obvious. No previous quantitative studies in this direction have been possible, however, since suitable means of evaluating this property has not been available. The development of the instrumented tablet machine (2, 3) has opened the way to precise evaluation of lubricating efficacy of a given material. Thus, the relationships that exist between chemical structure and lubricating properties can be extensively studied.

Two types of data yielded by the instrumented machine can be used in evaluating the lubricating properties of tested materials: (*a*) the ratio of maximum lower to maximum upper punch force of compression, designated *R*, and (*b*) the force required to eject the tablet from the die.

The ratio, *R*, would approach unity if perfect lubrication could be achieved. This ratio varied somewhat with the compressional force level employed; results given in this report were taken at relatively constant compression forces. The force required to eject the finished tablet from the die would, hypothetically, approach zero under perfect lubrication conditions.

The types of lubricants are denoted on the basis of publications concerning lubricants and lubrication (6, 7, 8). Fatty acids and their salts have all been considered as boundary-type lubricants while hydrocarbons and waxes were classed as fluid-type lubricants in accordance with those examples found in the above mentioned reports.

Table III gives the results of this study where 2% lubricant was applied to the test granulations. All *R* values given were the average of at least five separate determinations, and the ejection forces represent the median of the same number of determinations. The data are discussed in the following sections in terms of the chemical structures of the materials studied as tablet lubricants.

Hydrocarbons.—The experimental data suggest that the effectiveness of hydrocarbon lubricants is

TABLE II.—RESULTS OF COATING THE DIE WALL WITH BOUNDARY-TYPE LUBRICANTS FOLLOWED BY COMPRESSION OF SUCCESSIVE TABLETS OF NONLUBRICATED SODIUM BICARBONATE TEST GRANULATION[a]

Lubricant Used to Coat Die Wall		Tablet Number 1	2	3	4	5	6	7	8
Magnesium stearate	*R* Value	0.95	0.91	0.90	0.86	0.80	0.70	0.72	0.68
	Ejection force, Kg.	6	8	10	20	43	65	88	103
Stearic acid, 100-mesh	*R* Value	0.93	0.91	0.84	0.86	0.80	0.82	0.71	0.67
	Ejection force, Kg.	9	10	10	20	40	60	90	108

[a] Tablet No. 1 represents maximum lubrication attainable. The value *R* is the ratio of lower to upper punch forces at maxmium compression.

TABLE III.—RESULTS OF EVALUATING DIFFERENT COMPOUNDS AS TABLET LUBRICANTS[a]

Material Used as Lubricant	Sulfathiazole Granulation		Sodium Bicarbonate Granulation		Type of Lubricant Mechanism
	R Value	Ejection Force, Kg.	*R* Value	Ejection Force, Kg.	
Perfect lubrication (hypothetical)	1	0	1	0	..
Control (no lubrication)	0.67	110–170	0.60	150–200	..
Magnesium stearate	0.95	8	0.93	9	B
Paraffin[b]	0.96	13	0.90	32	F
Calcium stearate	0.94	10	..	..	B
Zinc stearate	0.94	14	..	..	B
Sodium elaidate	0.93	8	0.92	4	B
Duponol C	0.93	25	0.92	10	B
Sodium oleate	0.92	9	0.91	4	B
Sodium laurate	0.91	10	0.90	5	B
Sodium myristate	0.91	10	0.90	6	B
Sodium stearate	0.91	16	0.89	8	B
Distearin	0.91	21	..	..	F
Myristic acid[b]	0.91	30	..	..	B
Aluminum stearate	0.90	18	..	..	B
Elaidic acid	0.90	21	0.92	8	B
Glycerol monostearate	0.90	28	..	..	I
Sarkosyl NL 100 (Geigy)	..	..	0.91	8	?
Stearic acid	0.90	30	0.91	11	B
Stearyl alcohol[b]	0.90	40	..	..	F?
Alrowax (Geigy)	..	..	0.90	10	F
Cetyl alcohol	0.90	40	0.89	30	F?
Glycerol monopalmitate	0.89	22	..	..	I
Beeswax (white)	0.89	24	..	..	F
Ceresin (white)	0.89	22	..	..	F
Magnesium lauryl sulfate	0.88	24	..	..	B
Stearyl alcohol	0.87	32	0.90	26	F?
Myristic acid	0.87	38	0.91	10	B
Cetyl alcohol[b]	0.87	50	..	..	F?
Lauroyl isopropanol amide (Geigy)	..	..	0.87	16	B
Carbowax 4000 (Carbide and Carbon Chemicals Co.)	0.86	38	0.86	44	I
Arlacel 60 (sorbitan monostearate) (Atlas)	0.85	31	..	..	I
Ethofat 60/60 (Armour)	0.85	44	..	..	I
Talc U. S. P.	0.85	45	0.76	70	A
Lauric acid	0.83	40	0.92	16	B
Graphite	0.83	53	..	..	B
Polyvinyl myristate	0.82	38	..	..	I
Aldo 33 (Glyco Products)	0.82	50	..	..	F
Polyvinyl stearate	0.81	30	0.85	20	I
Propylparaben	0.80	70	..	..	F
Myristyl alcohol	0.79	55	0.76	67	F?
Mineral oil, heavy U. S. P.[b]	0.78	44	0.70	80	F
Ethofat C/60 (Armour)	0.78	64	..	..	I
Diglycol stearate S (Glyco Products)	0.78	80	..	..	I
Zein (isolated free protein)	0.77	74	..	..	A
Propylparaben[b]	0.77	80	..	..	F
Boric acid	0.76	90	..	..	A
Acetophenetidin	0.75	85	0.78	73	A
Peanut protein	0.73	112	..	..	A
Sodium benzoate	0.72	103	..	..	?
Lauryl alcohol[b]	0.71	71	0.64	85	F
Benzoic acid	0.71	100	..	..	?
Sodium chloride	0.70	100	..	..	?
Starch 5% (corn)	0.70	130	0.64	150	?
Starch 2% (corn)	0.68	135	0.69	110	?
Calcium citrate	0.69	107	..	..	?
Soybean protein	0.67	127	..	..	?
Control	0.67	110–170	0.60	150–200	..
Hydrocinnamic acid	0.67	137	..	..	O
Super-Floss (Johns-Manville)	0.67	141	..	..	O
Ethomid HT/60 (Armour)	0.66	143	..	..	O
Anthracene	0.66	166	..	..	O
Aluminum hydroxide	0.66	175	..	..	O
Calcium acetate	0.65	120	..	..	O
Zirconium oxide	0.65	158	..	..	O
Vermiculite (325 mesh, micronized)	0.63	182	0.65	126	O
Aluminum oxide	0.63	194	..	..	O
Magnesium trisilicate	0.63	200	..	..	O
Zirconium silicate	0.63	200	..	..	O

TABLE III (*Continued*)

Material Used as Lubricant	Sulfathiazole Granulation — *R* Value	Sulfathiazole Granulation — Ejection Force, Kg.	Sodium Bicarbonate Granulation — *R* Value	Sodium Bicarbonate Granulation — Ejection Force, Kg.	Type of Lubricant Mechanism
Pectin	0.62	205	..	..	O
Silene E. F. (Pittsburgh Plate Glass Co.)	0.62	225	..	..	O
Veegum (Vanderbilt)	0.62	265	0.61	147	O
Carbowax 4000 (Carbide and Carbon Chemicals Co.)[b]	0.61	120	..	..	I
Mucic acid	0.61	293	..	..	O
Silicone resin (XR-102, very fine powder D.C.)	0.60	235	..	..	O
Calcium gluconate	0.59	291	..	..	O
Silica gel (0–6 mμ, very fine, grade 979, D.C.)	0.58	280	..	..	O

[a] *R* = Maximum lower punch force/maximum upper punch force. Lubricants were usually added to the granulation as 100-mesh or finer powders. 2% Lubricant was used. Three-eighths inch flat-faced steel punch and die set was used. Tablets of sulfathiazole weighed 0.380 Gm. and sodium bicarbonate tablets weighed 0.480 Gm. The difference in weight of the tablets is accounted for by the difference in density of the granulations. All tablets prepared were approximately the same size. The machine settings were essentially the same for all these determinations. Designations of the types of lubricants are: F—fluid type, B—boundary type, I—indefinite mechanism of action, A—antiadherent, O—no apparent lubricant activity, ?—lubrication mechanism questionable.

[b] Lubricants added as ethereal solution (exception—Carbowax 4000 added as methanol solution).

dependent largely on their relative viscosity. This is especially evident in Fig. 1, where both ceresin and paraffin, which possess the highest viscosity, appear to be the best lubricants in this group. Mineral oil, on the other hand, apparently does not have sufficient viscosity to lubricate satisfactorily tablets under conditions similar to those employed in these compressions.

Soaps.—The metallic salts of fatty acids generally exhibit excellent lubricating properties. Figure 3 reveals that there appears to be no appreciable loss in lubricating activity of sodium salts of saturated, straight chain fatty acids as the carbon chain length is decreased from 18 to 12 carbon atoms. The polyvalent metal salts of these acids appeared to be superior to the monovalent sodium salts. Sodium oleate and its trans form, sodium elaidate, showed essentially the same lubricant activity.

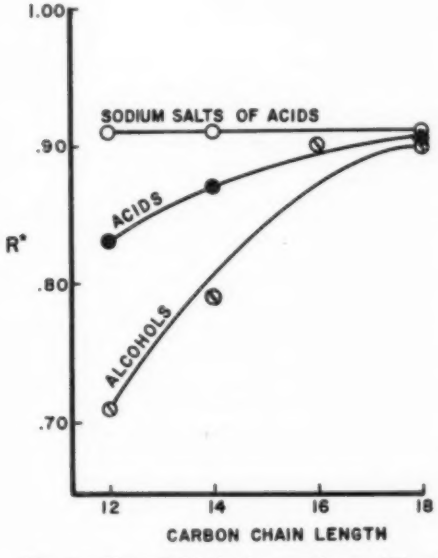

Fig. 3.—Plot of lubricating efficiency (*R*) of saturated alcohols, acids, and sodium salts of acids *versus* the number of carbon atoms in the molecule. All lubricants were added as 100-mesh powder in 2% strength. *R* = Maximum lower punch force/maximum upper punch force.

Fatty Acids.—The saturated straight chain fatty acids are also excellent lubricants although generally they are not so effective as their corresponding salts. The lubricating properties of stearic, myristic, and lauric acids when applied as 100-mesh powders in 2% strength, decreased somewhat with decreasing carbon chain length as seen in Fig. 3. This relation has been encountered in other systems (9). Elaidic acid and stearic acid appeared to display approximately equal lubricating properties.

Fatty Alcohols.—Saturated straight chain alcohols generally appeared to exhibit properties of fluid type lubricants, which may be expected because of their weak polarity. The decrease of lubricating properties as carbon chain length is diminished (Fig. 3) appears to correspond closely to decrease in viscosity of the alcohols used. Thus, the longer carbon chain, waxy alcohols may serve as satisfactory tablet lubricants.

Alkyl Sulfonates.—Duponol C (sodium lauryl sulfate) showed excellent lubricating activity while magnesium lauryl sulfate appeared to be a good tablet lubricant. On the basis of results obtained from the evaluation of salts of fatty acids as tablet lubricants, magnesium lauryl sulfate could be expected to be equal to or even superior to sodium lauryl sulfate as a tablet lubricant. However, as seen in Table III sodium lauryl sulfate has significantly greater lubricating properties than the magnesium derivatives.

Oxides and Silicaceous Materials.—This group includes such materials as talc, boric acid, aluminum oxide, zirconium silicate, silicone resins, and vermiculite. From the results of this study and by virtue of their physical and chemical nature, compounds of this group appear to be antiadherents rather than lubricants in the more restricted meaning of the term. In view of the slippery, impalpable quality of such substances as mentioned above, the findings which indicated that these compounds may accentuate the granule-die wall friction was somewhat surprising. It would seem that forces employed in tableting procedures are such that the beneficial effects of this type compound may not be exerted.

Salts.—Neither metallic salts of lower molecular weight organic acids, such as benzoic or acetic acid, nor inorganic salts have shown appreciable lubricating properties.

Miscellaneous.—Polyvinyl and polyethylene glycol derivatives showed considerable lubricating properties, although there was much variation from one compound to another. Carbohydrates such as starch, pectin, and mucic acid appear to have no value as lubricants. Proteins, e. g., zein, peanut, and soybean protein exhibited no appreciable lubricating activity, however, they appeared to act as antiadherents in some measure. The amides, Sarkosyl NL 100 (Geigy) and lauroyl isopropanol amide, displayed lubricant activity on the order of that of fatty acids. Although only two compounds were used here it would appear probable that long chain amides could exhibit sufficient polarity to function as boundary-type lubricants.

In view of the results of this study and the fundamental nature of lubrication it does not appear likely that inorganic salts, carbohydrates, or other highly water-soluble compounds can possess the properties necessary to be good tablet lubricants.

CONCLUSIONS

The conclusions which may be drawn on the basis of this study are:

1. Tablet lubrication appears to be generally amenable to the theory of lubrication previously reported for other systems.

2. The effectiveness of a hydrocarbon lubricant under the conditions of this work seems to depend on its viscosity.

3. The lubricating properties of a boundary-type lubricant are generally related to the carbon chain length of the dipolar molecule.

4. The dipolar, boundary-type lubricants appear to be superior to the type that depends on viscosity for its lubricant properties.

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Sulfaethylthiadiazole VI. Blood and Urine Concentrations from Sustained and Immediate Release Tablets*

By ARNOLD E. NICHOLSON, STANLEY J. TUCKER, and JOSEPH V. SWINTOSKY

In a cross-over study employing six adult human subjects, blood concentrations and urinary elimination patterns were determined following ingestion of sustained and quick release sulfaethidole tablets. Data are presented which indicate that the sustained release tablets give less variation between maximum and minimum blood concentrations and more uniform urinary excretion rates than the quick release tablets. A convenient and safe twelve-hour dosage schedule is therefore made possible with the sustained release tablets. The use of the specific velocity constant for drug elimination, or biologic half-life, in the selection of regimens and the development of dosage forms is illustrated.

MEASUREMENTS of blood concentrations and urinary excretion rates are useful in determining time-dose relationships in the study of some chemotherapeutic agents. For example, during the first eight to twelve hours after administration of therapeutic doses of sulfaethidole (sulfaethylthiadiazole, SKF, or SETD) and some related sulfonamides, measurements by blood concentrations of elimination from the human tissues may be described conveniently in terms of a first-order rate expression (1–11). Blood concentrations vary according to the route of administration, the dosage form, and the size of the dose. Furthermore, determinations of blood concentrations enable the pharmacist to compare the properties of various formulas and to select the formula which best fulfills the objectives sought in a new product.

In developing a new tablet form of SETD, one pharmaceutical objective was to formulate a tablet which, in clinical usage, would establish therapeutically effective blood concentrations within one hour after administration, and main-

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tain them more uniformly for twelve hours than is possible with a conventional tablet. When taken every twelve hours, a sustained release dosage form eliminates the necessity of interrupting nighttime sleep and makes possible a convenient twice-daily dosage schedule. Secondly, the new tablet should maintain relatively uniform urinary excretion between doses to assure effective concentrations of SETD in the treatment of urinary tract infections. And, finally, this form should reduce peak blood concentrations and kidney loads of the drug appreciably below those observed with an equivalent dose of the quickly-absorbed form of the drug. Although the occurrence of crystalluria or hematuria in human subjects receiving large doses of SETD is rare (12), it seemed desirable to reduce still further the possibility of such complications during therapy.

This paper outlines the formulation procedures that fulfill these objectives and describes a cross-over study that illustrates the differences in the blood and urine elimination patterns resulting from administration of SETD in quickly disintegrating tablets and in sustained release tablets.

EXPERIMENTAL

Plain SETD Tablets.—By means of conventional procedures we prepared quickly disintegrating tablets that measured one-half inch in diameter and had a flat face and beveled edge. In addition to 11.5% starch as disintegrant, each tablet contained 0.65 Gm. SETD and disintegrated within fifteen minutes in the standard U. S. P. disintegration test employing artificial gastric fluid.[1]

Special SETD Tablets.—A two-stage, compression-type, rotary tablet press was employed to make two-layered tablets of the same shape and size as the plain tablets (13). The top layer of each tablet contained 0.38 Gm. of SETD formulated to disintegrate within fifteen minutes after oral ingestion. The bottom layer, which contained 0.27 Gm. of SETD, was formulated to release its drug gradually after ingestion. The standard U. S. P. disintegration test showed that the time required for complete disintegration in artificial gastric fluid exceeded five and one-half hours. In artificial intestinal fluid disintegration time exceeded four hours.

Clinical Studies.—Six healthy young adult subjects were used for clinical tests. On the first test day three subjects received 3.9 Gm. of SETD in plain tablets, and three received the same amount in sustained release tablet form. Two weeks later each subject received the alternate form. Venous blood samples were withdrawn from each subject at one, two, three, six, nine, twelve, fifteen, eighteen, and twenty-four hours after administration. Urine samples were collected after three, six, nine, twelve, fifteen, eighteen, twenty-four, forty-eight, and seventy-two hours. Each subject ingested 200 ml. of water at zero, three, six, nine, twelve, fifteen, eighteen, and twenty-four hours. Within an hour after drug ingestion subjects were permitted to ingest food and fluid as desired.

Concentrations of the free and of the total (free plus conjugated) SETD in the blood and urine were determined by a modification of the macromethod described by Frisk (14). Colorimetric measurements of the final solutions were made with an Evelyn colorimeter at a wavelength near 550 mμ. Comparisons were made with appropriate standards for dilutions and origin of samples.

RESULTS AND DISCUSSION

Blood Concentrations.—There were no problems in administering either tablet form. Blood data indicated that per cent of SETD conjugated was very nearly identical for both types of tablets, varied little between individual subjects, and was of a very low order. These results conform with previously published results (5–9).

The data from Table I, reflecting the average free SETD blood concentrations, are plotted in Fig 1.

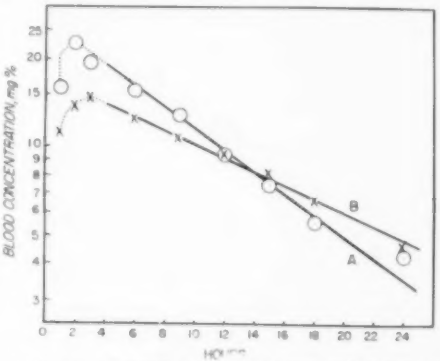

Fig. 1.—Average free SETD concentrations in the blood for six human subjects receiving single 3.9-Gm. dose of (*A*) plain SETD tablets and (*B*) sustained release SETD tablets.

With the plain tablets the average peak concentration, 22 mg. %, occurred two hours after ingestion and decreased exponentially up to twenty-four hours. The slope of the straight line indicates an average specific velocity constant for elimination from the blood, k_b, of 0.09 hour^{-1}. This average value agrees well with that determined on a larger group of subjects receiving the plain drug in 1–4-Gm. oral doses (6). The data of Table I and Fig. 1 indicate that at a dosage of 3.9 Gm. the drug is usually absorbed within two to three hours from the quickly disintegrating tablets. The remainder of the drug is eliminated from the blood at an average rate of about 9% per hour.

Absorption of the quickly released portion of the sustained release tablets is also rapid. After one, two, and three hours the average blood concentrations are 11, 14, and 15 mg. %, respectively. The excessively high peak values observed following administration of the plain tablet were not seen with the two-layered sustained release tablet. Furthermore, although the average blood concentrations appear to decrease exponentially, the rate of decrease is slower than that observed with the plain tablets.

[1] Disintegration disks were not employed in these studies.

TABLE I.—FREE AND FREE PLUS CONJUGATED (TOTAL) SETD BLOOD CONCENTRATIONS (MG. %) FOR SIX SUBJECTS RECEIVING 3.9-GM. ORAL DOSES IN PLAIN AND SUSTAINED RELEASE TABLETS

Subject	Sex	Weight, Kg.	Age, Yr.	Hour	Plain Tablet		Sustained Release Tablet	
					Free	Total	Free	Total
Nn	M	79.5	29	1	13.3	13.8	1.5	2.0
				2	23.1	24.0	7.0	7.2
				3	24.0	24.8	12.9	13.3
				6	26.5	27.0	12.1	12.5
				9	26.0	26.5	12.1	12.4
				12	14.9	15.4	11.6	11.9
				15	11.6	11.9	8.3	8 8
				18	9.8	10.1	6.4	6.6
				24	7.2	7.2	4.6	5.0
Lo	M	79.5	24	1	27.5	28.2	14.5	14.9
				2	27.0	27.5	14.1	14.5
				3	19.0	21 0	13.8	14.1
				6	13.3	13.8	12 9	13.3
				9	9.0	9.7	11.0	11.2
				12	8.3	8.8	9.0	9.3
				15	6.6	6.9	8.3	8.8
				18	4.6	4.9	7.0	7.2
				24	4.1	4.4	5.0	5.0
Sk	M	86.4	23	1	19.5	21.0	11.2	11.6
				2	19.0	19.5	16.4	17.0
				3	19.0	19.5	14.5	14.9
				6	13.3	13.8	12.5	12.9
				9	11.9	12.1	10.3	10.6
				12	8.8	8.9	9.3	9.6
				15	7.8	8.0	7.8	8.0
				18	6.6	6.9	6.0	6.2
				24	4.4	4.6	3.4	3.6
Mr	M	88.6	23	1	8.2	8.2	11.0	11.2
				2	12.3	13.8	12.5	12.9
				3	13.8	14 1	11.9	12.1
				6	12.1	12.5	8.8	8.9
				9	9.3	9.6	7.8	8.0
				12	7.5	7.8	7.8	8.0
				15	7.0	7.2	6.6	6.9
				18	4.6	4.9	5.1	5.8
				24	4.4	4.6	5.0	5.1
Gs	M	65.9	35	1	12.9	13.3	12.1	12.5
				2	23.1	24.0	15.9	16.4
				3	16.4	17.0	17.0	17.4
				6	9.0	9.3	12.5	12.9
				9	7.2	7.5	10.1	10.3
				12	7.2	7.6	8.0	8.2
				15	6.2	6.4	7.5	7.8
				18	2.4	2.4	6.9	7.0
				24	2.0	2.0	4.1	4.4
Ve	F	55	26	1	12.9	10.1	14.5	14.9
				2	26.0	26.5	15.4	15.9
				3	18.4	19.0	16.4	17.0
				6	15.9	16.4	12.9	13.3
				9	11.2	11.6	10.3	10.6
				12	8.2	8.2	9.6	9.8
				15	4.6	4.6	8.0	8.2
				18	4.1	4.1	6.9	7.0
				24	2.9	3.1	4.1	4.4

The slope of the line is 0.05 hour^{-1}. This smaller slope indicates that in the sustained release tablet the drug continues to be absorbed from the gastrointestinal tract for a considerable period and partially offsets the decrease in blood concentrations that results from the normal physiologic excretion. Although much higher blood concentrations occurred in the early hours after administration of quickly disintegrating SETD tablets, the blood concentration curves cross after twelve to fourteen hours.

The fact that the blood concentration curves in Fig. 1 cross after twelve to fourteen hours indicates that absorption from the sustained release tablet persists for an appreciable time beyond that of the plain tablet. Although the plain tablet is capable of maintaining a therapeutically effective blood level throughout a twelve-hour period, it does this by rapidly attaining a rather high initial blood concentration.

The magnitude of the spread between maximum and minimum blood concentrations that results from administration of identical doses of SETD is greatly reduced in the zero to twelve-hour time interval by the sustained release tablet. In this time interval it may be noted that the blood concentration spread resulting from administration of the plain tablet is about twice as great as for the sustained release tablet, yet the twelve-hour blood concentrations are virtually identical. Thus the sustained release tablet maintains effective therapeutic concentrations

in the blood for a twelve-hour period and yet avoids the excessively high initial blood concentrations that might increase the possibility of toxic effects.

Urinary Excretion.—Tablet design has no effect on the per cent of the drug that is biotransformed. The urinary data in Table II show that, over a seventy-two-hour period, the average biotransformation of SETD administered in plain tablets was 7.2%; it was 7.1% for the sustained release tablet. These figures correspond closely to those reported previously (6–9).

Figures 2 and 3 compare the two tablet dosage forms on the basis of milligrams of free, therapeutically active drug excreted in the urine. These data bear a relationship to the blood concentration data. The sustained release tablets reduce the load on the kidney and decrease the rapid loss of the drug that is seen during the first nine hours following adminis-

TABLE II.—FREE AND FREE PLUS CONJUGATED (TOTAL) SETD URINARY EXCRETION (MG.) FOR SIX SUBJECTS RECEIVING 3.9-GM. ORAL DOSES IN PLAIN AND SUSTAINED RELEASE TABLET FORM

Subject	Collection Time Interval, Hr.	Plain Tablet		Sustained Release Tablet	
		Free	Free Plus Conjugated	Free	Free Plus Conjugated
Nn	0–3	382	411	144	144
	3–6	457	473	470	470
	6–9	342	352	203	219
	9–12	475	482	255	269
	12–15	420	476	378	398
	15–18	248	260	276	290
	18–24	257	257	289	302
	24–48	700	798	720	738
	48–72	117	118	732	806
	Total	3,398	3,627	3,467	3,636
Lo	0–3	1,164	1,197	623	668
	3–6	872	872	688	762
	6–9	449	485	468	504
	9–12	296	319	437	444
	12–15	217	264	416	448
	15–18	149	193	216	232
	18–24	231	284	220	249
	24–48	130	130	128	132
	48–72	132	149	136	136
	Total	3,640	3,893	3,332	3,575
Sk	0–3	708	792	440	460
	3–6	739	764	808	808
	6–9	710	730	448	462
	9–12	374	396	528	560
	12–15	204	247	475	489
	15–18	159	198	187	208
	18–24	204	220	299	352
	24–48	396	426	480	480
	48–72	0.0	0.0	20	23
	Total	3,494	3,773	3,685	3,842
Mr	0–3	704	809	625	625
	3–6	658	712	563	563
	6–9	434	448	417	473
	9–12	282	288	460	483
	12–15	275	275	324	324
	15–18	117	123	165	180
	18–24	381	417	232	242
	24–48	357	372	233	275
	48–72	206	221	241	294
	Total	3,414	3,665	3,260	3,459
Gs	0–3	545	545	312	380
	3–6	1,036	1,123	320	368
	6–9	364	371	248	270
	9–12	193	206	640	742
	12–15	280	317	225	246
	15–18	119	119	302	341
	18–24	117	134	274	284
	24–48	109	146	91	152
	48–72	195	293	97	94
	Total	2,958	3,254	2,509	2,877
Ve	0–3	558	558	594	612
	3–6	857	911	459	459
	6–9	764	781	126	145
	9–12	381	439	343	417
	12–15	280	280	264	308
	15–18	101	130	312	352
	18–24	240	300	301	301
	24–48	200	220	268	333
	48–72	206	243	110	110
	Total	3,587	3,862	2,777	3,037

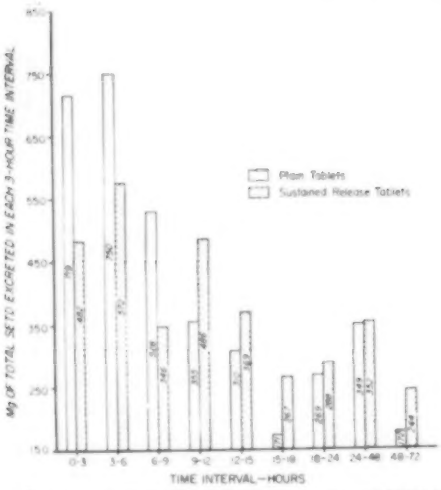

Fig. 2.—Average urinary excretion of total SETD for six human subjects receiving 3.9-Gm. oral dose of plain and sustained release tablets.

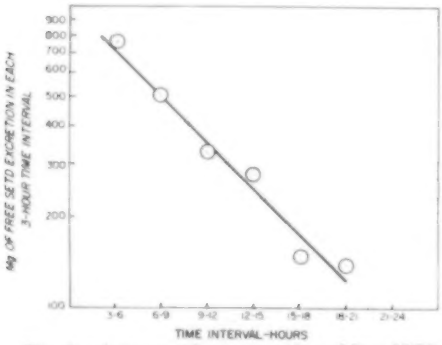

Fig. 3.—Average urinary excretion of free SETD for six human subjects receiving 3.9-Gm. oral dose of plain tablets.

tration of the plain tablets. After nine hours, SETD collected in the urine from the sustained release tablets exceeded that observed for the plain tablets. The sustained release tablets make it possible to maintain more uniform urinary excretion rates and more uniform blood concentrations of SETD during the zero to twelve-hour time interval.

The seventy-two-hour urinary collection data show that a minimum of 94% of the drug in the plain tablet is absorbed. By contrast, an average of 87% of the original dose of the sustained release tablet was accounted for in the urine. From Fig. 2 it would appear that this small difference would be accounted for if the collection period had been continued.

Figure 3 shows the average amount of free SETD excreted in each three-hour interval (from zero to twenty-one hours) after administration of the plain tablets, plotted (3, 7) according to the equation:

$$\log \frac{\Delta C}{\Delta t} = \frac{-k_{el}}{2.303} + K$$

The fairly straight line resulting from plotting the average urinary excretion data indicates a biologic half-life of about six hours for SETD in these subjects. This figure agrees fairly closely with the average of eight hours determined from the blood concentrations in these same subjects and is within the range reported previously for similar subjects (6–9).

SUMMARY AND CONCLUSIONS

The study illustrates how the use and safety of therapeutically active drugs may be increased by the application of pharmaceutical formulation techniques, and a method of evaluating two dosage forms in terms of biologic half-life or velocity constant for drug elimination. Since the sustained release tablet form of sulfaethidole provides prompt and prolonged effective blood and urine concentrations of the drug, safe and effective therapy can be maintained on a schedule of two doses a day (q. 12h.). The lower peak blood concentrations and kidney loads which result from the use of the sustained release tablet decrease the possibility of toxic effects. This approach to the design of dosage forms and regimens appears applicable to a number of other drugs.

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An Analysis of the Volatile Oil of *Lomatium Suksdorfii**

By FRANK A. PETTINATO†, LOUIS FISCHER, and NATHAN A. HALL

The volatile oil of the fruits of *Lomatium Suksdorfii* (Wats.) Coulter and Rose, obtained by steam distillation, was found to consist of over 90 per cent terpene hydrocarbons among which α- and β-pinene, *d*-limonene, and *d*-β-phellandrene were identified. Isovaleric acid was identified as a component of the oil, and both acetic acid and isovaleric acid were found in the aqueous portion of the steam distillate.

THE RESULTS of a preliminary phytochemical and biological study of *Lomatium Suksdorfii* (Wats.) Coulter and Rose, as well as the physical and chemical characteristics of the volatile oil obtained from the fruits by steam distillation, have been reported (1). As an extension of this work a more detailed study of the oil and the aqueous portion of the steam distillate obtained during the separation of the oil was undertaken.

EXPERIMENTAL

Volatile Acids of the Aqueous Distillate.—The aqueous distillate from the steam distillation of the fruit was found to contain from 5 to 7 meq. of acid per liter. Therefore, an attempt to separate and identify these acids was made.

A 5 × 30-cm. chromatography tube was filled to 17 cm. with previously soaked and washed Amberlite IRA 400 anion exchange resin (Rohm & Haas Co.). The resin was first treated with 200 ml. of 1 *N* NaOH and the excess base was washed from the column with distilled water until the effluent was neutral to phenolphthalein. The filtered, aqueous distillate was allowed to pass through the column at the rate of 30 ml. per minute. As the acids combined with the dark brown basic resin, it became light tan in color, thus the exchange process could be followed visually. When the light tan front approached the bottom of the resin bed (usually after 12 to 16 L. of distillate), the column was washed with 4 L. of distilled water and the acids were eluted as sodium salts with 200 ml. of 1 *N* NaOH. The column was then washed with 1.5 to 2 L. of water (flow rate 8–10 ml. per minute) until the washings were neutral to phenolphthalein. In this manner, ten liters of effluent was collected from 125 L. of the distillation waters.

The effluent was filtered and concentrated to 300 ml. in a rotary film evaporator under reduced pressure (water aspirator) at 50–60°. The concentrate was acidified with 36% HCl and extracted with ethyl ether for thirty hours in a liquid-liquid continuous extraction apparatus. After the ether was allowed to evaporate spontaneously, 25 ml. of a brownish-orange liquid remained and possessed the characteristic odor of the low molecular weight fatty acids.

Both the rapid, horizontal paper chromatographic method of Roberts and Bucek (2) and the descending paper chromatographic method of Manganelli and Brofazi (3) were used to obtain a preliminary characterization of the unknown volatile acids. These two methods utilized a butanol-water solvent system in which the acids were separated as their ethylamine salts. After development, the acid spots were located by dipping the papers in a 0.2% solution of either bromophenol blue or chlorophenol red in 95% ethanol. Comparison of the chromatograms of the unknown volatile acids with those of a known mixture of C_2 to C_5 fatty acids indicated the presence of acetic, propionic, butyric, and isovaleric acids. Acetic and isovaleric acids were most prominent, while butyric acid was present in considerably smaller concentration. A spot corresponding to propionic acid appeared on the paper only when high concentrations of the unknown acid mixture were used.

Butyric and isobutyric acids could not be separated from known acid mixtures by either paper chromatographic method used. Valeric and isovaleric acids were readily separated from known mixtures by the descending but not by the horizontal method.

Separation and Identification of Acetic Acid.—A partial separation of acetic acid from the other acids in the mixture was achieved by extracting an ether solution with a saturated sodium chloride solution. Chromatography of the extract by the rapid, horizontal method showed acetic acid as the predominate constituent. The saline extracts were shaken with five separate 25-ml. portions of ether which were not combined. Separate chromatograms showed that the last three ether extracts contained only acetic acid. These three extracts were combined, the ether allowed to evaporate spontaneously, and the mixture neutralized with 1 *N* NaOH. When the mixture was evaporated to dryness on a steam bath, 960 mg. of the sodium salt of the acid was recovered. From 300 mg. of the salt, 400 mg. of the anilide (m. p. 114–115°) was prepared by the method of Cheronis and Entrikin (4). The melting point was not depressed by an authentic sample of acetanilid.

Separation and Identification of Isovaleric Acid.—The column chromatographic method of Moyle, Baldwin, and Scarisbrick (5) was used to separate isovaleric acid from the volatile acid mixture. The solvents, 1, 10, and 30% solutions of butanol in chloroform, were equilibrated with 2 *M* K_2HPO_4. One hundred grams of Mallinckrodt silicic acid, 100 mesh, specially prepared for chromatography, was used, without prior treatment, as the adsorbent. The column was buffered with a 2:1 mixture of 2 *M* K_2HPO_4 and 2 *M* KH_2PO_4.

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† Fellow of the American Foundation for Pharmaceutical Education. Present address: School of Pharmacy, Montana State University, Missoula.

The volatile acid mixture, 0.4 ml. (which had been dried over anhydrous sodium sulfate and filtered) was dissolved in 20 ml. of 1% butanol in chloroform, placed on the column, and elution was begun with the same solvent. Twenty-five-ml. fractions of the eluate were collected and 5-ml. aliquots of each fraction were titrated with 0.01 *N* methanolic KOH. When the titer of an aliquot of a fraction indicated that no appreciable quantity of acid was being eluted, the eluting liquid was changed to one containing more butanol. Isovaleric acid was eluted with 1% butanol in chloroform, other acids by 10% butanol and acetic acid by 30% butanol. To examine a major fraction for the presence of a particular acid, 10 ml. of the eluate was shaken with three 2-ml. portions of 33% aqueous ethylamine solution, and 3 to 5 microliters of the ethylamine extract were chromatographed on paper by the horizontal method.

From the operation of several columns, fractions were collected containing approximately 9 meq. of isovaleric acid. After the combined fractions were nearly neutralized with 1 *N* NaOH and evaporated to dryness, 860 mg. of the salt was recovered. The *p*-toluidine derivative (4) prepared from 200 mg. of the salt melted at 105–107° and the melting point was undepressed by authentic isovalero-*p*-toluidide. The anilide prepared from 300 mg. of the salt melted at 110–111° and the melting point was not depressed by authentic isovaleranilid.

Attempted Identification of Other Acids.—Paper chromatography of a few fractions showed the presence of an acid with an R_f value corresponding to butyric acid. Attempts to prepare a piperazine derivative (6) and toluidine derivative (4) were unsuccessful. Although the presence of propionic acid was indicated on some chromatograms, the amounts present were too small to permit characterization.

Free Acids of the Oil.—The free acids were extracted from 500 ml. of the volatile oil with 5% Na_2CO_3. The carbonate solution was acidified with HCl and extracted with ethyl ether. After the concentrated ether solution was dried over anhydrous sodium sulfate, a portion was subjected to the paper chromatographic methods discussed in the characterization of the aqueous distillate acids. The extract gave a spot with an R_f value corresponding to that of isovaleric acid. The presence of another acid, which appeared as a diffuse spot on the solvent front, was indicated but since the spot could not be reproduced consistently, no identification was attempted.

Phenols.—After the acids were removed, the oil was extracted with 5% NaOH solution, the solution was acidified with HCl, and the phenols were extracted with ethyl ether. On evaporating the ether, a small amount of residue remained which gave a reddish-violet color with ferric chloride test solution, indicating the presence of phenolic constituents.

Aldehydes and Ketones.—After the acids and phenols had been removed, the oil was extracted with saturated sodium sulfite solution. The sulfite solution was made alkaline (pH 10) with saturated Na_2CO_3 solution and extracted with ether. Evaporation of the ether left a small quantity of nearly colorless residue which gave a positive Schiff's and 2,4-dinitrophenylhydrazine test, indicating the presence of aldehydes and/or ketones.

Separation of the Terpenes and Oxygenated Constituents.—After the oil had been treated to remove the acids, phenols, and carbonyl constituents, it was examined for oxygenated constituents by the silicic acid chromatostrip technique of Kirchner, Miller, and Keller (7) with petroleum ether as the developing solvent and the fluorescein-bromine detection test. The chromatostrips revealed that the extraction procedure had not removed all of the oxygenated constituents, so the oil was separated chromatographically on a silicic acid column into a hydrocarbon and oxygenated fraction by the method of Kirchner and Miller (8). The terpene fraction was eluted with petroleum ether and the oxygenated fraction with ethyl acetate. After the solvents were removed under reduced pressure, the terpene fraction from 425 ml. of oil was 390 ml. or 91.8% of a colorless, pleasant-smelling liquid and the oxygenated fraction was 15.5 ml. or 3.6% of a yellow, viscid liquid with a characteristic, unpleasant odor. About 5% of the oil was lost in the separation process. The terpene fraction was found to be essentially free of oxygenated constituents by the chromatostrip method.

Fractional Distillation of the Terpenes.—Three hundred eighty-five milliliters of the terpene portion was distilled at 10 mm. through a Todd precise fractionating assembly, model A, with a 1.2 × 90 cm. column packed with 4 mm. glass helixes and equipped with a Todd automatic reflux head. Approximately 90% of the material distilled in the 43–57° range. Five per cent was still-pot residue and the remaining 5% which was recovered from the dry-ice trap, was residual petroleum ether.

The distillate, 310 ml. (43–57° range) was fractionated through the Todd assembly equipped with the more efficient 0.5 × 90 cm. column containing a monel spiral. The distillation was again conducted at 10 mm. and a 10:1 reflux ratio and the density, refractive index, and the optical activity of the fractions were determined (see Table I).

Identification of α- and β-Pinene.—An α-pinene nitrosochloride was prepared from 1 ml. of fraction 1 by the method of Ehestädt (9). Thirty milligrams of the crude derivative was recovered which melted at 103–104° after recrystallization from a chloroform-methanol mixture. The melting point was not depressed by a nitrosochloride prepared from authentic *d*-α-pinene.[1]

Seven milliliters of fraction 3 and 10 ml. of fraction 4 were oxidized to nopinic acid with alkaline $KMnO_4$ by the technique of Wallach (10). About 10 mg. of nopinic acid (m. p. 128–129°) was separated and recrystallized from a benzene-hexane solvent. The melting point of nopinic acid obtained from fractions 3 and 4 was undepressed by nopinic acid prepared from *l*-β-pinene.[2]

Identification of *d*-β-Phellandrene.—A nitrosite derivative was prepared from 5 ml. of fraction 8 and 5 ml. of fraction 9 by the action of sodium nitrite and glacial acetic acid according to West (11). Approximately 450 mg. of impure derivative was recovered from each fraction. On recrystallization from a chloroform-methanol mixture, colorless needles were separated which melted at 92–93°

[1] Obtained from Delta Chemical Works, 23 West 60th St., New York, N. Y.

[2] Obtained from Delta Chemical Works, 23 West 60th St., New York, N. Y.

TABLE I.—PHYSICAL PROPERTIES OF THE FRACTIONALLY DISTILLED TERPENE FRACTION

Fraction	Boiling Range, °C.	Distillate, %	d_{25}^{25}	n_D^{20}	α_D^{25}
1	39–42	0.7	0.857	1.4686	+18.00
2	42–44	0.7	0.858	1.4718	21.00
3	44–46	2.5	0.854	1.4738	24.74
4	46–48	3.7	0.852	1.4750	26.95
5	48–50	2.0	0.848	1.4766	30.45
6	50–54	1.7	0.842	1.4781	34.80
7	54–55	3.8	0.838	1.4801	43.75
8	55–56	14.1	0.838	1.4812	45.80
9	56–57.5	69.8	0.840	1.4814	53.35
Residue and loss	...	1.0	...	...	...

Melting points of 102° for β-phellandrene-α-nitrosite and 97–98° for β-phellandrene-β-nitrosite have been reported (12). Repeated attempts to purify the nitrosite by recrystallization from chloroform-methanol, chloroform-butanol, and acetone-water failed to raise the melting point.

A nitrosochloride was prepared from 5 ml. of fractions 8 and 9 by the action of amyl nitrite and methanolic HCl by the method of West (11). Ninety milligrams of the nitrosochloride was recovered from fraction 8 and 55 mg. from fraction 9. After recrystallization from a methanol-water mixture, the nitrosochloride melted at 99.5–100°. The melting point of β-phellandrene-α-nitrosochloride has been reported to be 101–102° and that of β-phellandrene-β-nitrosochloride 100° (12). α-Phellandrene should not form a crystalline derivative (11).

A tetrabromide was prepared from 5 ml. of the same fractions by the method of Berry and MacBeth (13). Fraction 8 yielded 400 mg. of the tetrabromide which, after recrystallizing once from a chloroform-methanol mixture and twice from ethyl acetate, melted at 117–117.5°, $[\alpha]_D^{25}$ −57.6 (in $CHCl_3$). Fraction 9 yielded 400 mg. of the tetrabromide which melted at 117.5–118°, $[\alpha]_D^{25}$ −54.6 (in $CHCl_3$) after four recrystallizations from ethyl acetate. Berry and MacBeth (13) reported a melting point of 118–119° and $[\alpha]_D^{25}$ −53 for the *l*-tetrabromide derived from *d*-β-phellandrene.

Identification of *d*-Limonene.—A solution of 6 ml. of fraction 9 in a mixture of 5 ml. of amyl alcohol and 10 ml. of anhydrous ether was treated with 3 ml. of bromine in 3 ml. of anhydrous ether by the method of Gaponenkov (14). A solid tetrabromide separated after two weeks at −10°. After two recrystallizations from a chloroform-methanol solvent, 30 mg. of tetrabromide was recovered which melted at 105–106°, undepressed by authentic *d*-limonene tetrabromide.

The similarity in the physical constants of fractions 8 and 9 and the fact that both *d*-limonene and *d*-β-phellandrene had been identified in fraction 9 indicated that fraction 8 probably also contained a mixture of these two terpenes. Six milliliters of fraction 8 was treated according to the method of Davenport, Sutherland, and West (15) to eliminate the *d*-β-phellandrene. From 1 ml. of the resulting distillate 100 mg. of a tetrabromide was prepared according to the method of Gaponenkov (14). This tetrabromide melted at 105–106°, gave a specific rotation of +72.3 (in $CHCl_3$) and was identical with authentic *d*-limonene tetrabromide on a mixed melting point determination.

Ultraviolet Spectra of Fractions 8 and 9.—The ultraviolet spectra of both fractions (0.168 Gm./L. in 95% ethanol), determined with a Cary recording spectrophotometer, showed maximum absorption at 232 mμ which is in agreement with the value reported for β-phellandrene (15). The molecular extinction coefficient (ε) was 11,680 for fraction 8 and 11,600 for fraction 9. The ε for pure β-phellandrene has been reported as 19,300 (15), indicating that these two fractions consisted of about 60% β-phellandrene.

Attempted Characterization of the Oxygenated Portion.—The oxygenated portion (15.5 ml.) which had been separated from the terpenes on the silicic acid column, was fractionated through the Todd assembly at reduced pressure. Five fractions were collected but each distilled over such a wide range that characterization on the basis of physical constants was impractical. All the fractions gave a positive Schiff's and 2,4-dinitrophenylhydrazine reaction.

A 2,4-dinitrophenylhydrazone which was prepared from 0.13 ml. of the fraction which distilled between 95–100° at 5 mm. melted at 204–204.5° after recrystallization from an ethanol-ethyl acetate solvent. A semicarbazone prepared from 0.1 ml. of this fraction melted at 190–193° after three recrystallizations from ethanol-water. The yield was too small to permit further purification.

SUMMARY

A volatile oil was separated by steam distillation from the fruits of *Lomatium Suksdorfii* (Wats.) Coulter and Rose. Examination of the aqueous portion of the steam distillate by paper chromatography indicated the presence of acetic, isovaleric, propionic, and butyric acids; however, confirmation of only acetic and isovaleric acids was accomplished by the preparation of derivatives.

Isovaleric acid was found in the oil. The oil was separated by column chromatography into a terpene and an oxygenated fraction. The approximate amount of various constituents calculated from the fractions in which they were found to be present was as follows: *d*-α-pinene, 1%; *l*-β-pinene, 6%; *d*-limonene, 34%; and *d*-β-phellandrene, 50%. The oxygenated frac-

tion yielded an unidentified 2,4-dinitrophenylhydrazone and semicarbazone.

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The Preparation and the Properties of an Aspirin Homolog β-(*o*-Acetoxyphenyl)propionic Acid*

By CHARLES W. BAUER and EDWARD FRANCIS LASALA

A homolog of acetylsalicylic acid, β-(*o*-acetoxyphenyl)propionic acid, has been made successfully. Its toxicity and, analgesic properties have been determined on rats. It has been found to have analgesic properties in rheumatic patients. No free salicylic acid is eliminated in the urine. It appears that this compound does not exert its analgesic properties as a salicylate.

THE ANTIPYRETIC, analgesic, and antirheumatic effects of the salicylates in the human organism are well known. Clinically, it is equally well known that in the higher dosage levels of these drugs, tinnitus, deafness, diplopia, and occasional nausea and vomiting occur.

Whenever the chemical structure of the salicylates has been modified so as to eliminate the undesirable side effects, their therapeutic effectiveness has been reduced. Investigators soon learned the importance of the 1:2 relationship of the side chains. The *meta*- and *para*-isomers of salicylic acid are devoid of antipyretic, analgesic, and antirheumatic properties. Hence, one finds that most of the derivatives are truly salicylic acid derivatives in which the substitution of either the hydrogen or the hydroxyl radical of the carboxyl group or the substitution of the hydrogen of the phenolic group has been made. Theoretically, the most promising homologous series is the one in which the only alteration would be in the aliphatic acid side chain.

Repeated trials to synthesize the first higher homolog of acetylsalicylic acid, namely, *o*-acetoxyphenylacetic acid, have up till now, been unsuccessful. The next higher homolog, β-(*o*-acetoxyphenyl)propionic acid, has been synthesized and this paper deals with its proof of structure, physical and chemical properties, toxicity in mice, analgesic effects on rats, metabolism in human subjects and analgesic effects in arthritic patients.

EXPERIMENTAL DATA

Preparation of β-(*o*-Acetoxyphenyl)propionic Acid.—An adaptation of the method of Tiemann and Herzfeld (1) for the preparation of coumarin was used to prepare *o*-acetoxycinnamic acid, which was then hydrogenated to β-(*o*-acetoxyphenyl)propionic acid.

o-Acetoxycinnamic Acid.—A mixture of three parts of salicylaldehyde, four parts of anhydrous potassium acetate, and five parts of acetic anhydride was heated for one hour in a water bath. The mixture was then heated with a free flame to a temperature of 140° and was stirred until a homogeneous solution was obtained. The solution was cooled and treated with water. A yellow oil separated which was extracted with ether. The ether extract was extracted with a 5% solution of sodium carbonate. The alkaline aqueous solution was washed with two successive portions of ether and was then shaken for five minutes with activated charcoal. This mixture was filtered and the filtrate made acid to Congo red indicator with 6 *N* hydrochloric acid. A white crystalline precipitate of *o*-acetoxycinnamic acid was obtained. The dried precipitate had a melting point of 157° which corresponds to the melting point reported in the literature. A 12% yield was obtained.

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β-(o-Acetoxyphenyl)propionic Acid.—Twenty Gm. of the *o*-acetoxycinnamic acid was dissolved in 250 ml. of acetone, 0.5 Gm. of 10% palladium on charcoal was added as the catalyst, and the mixture was placed in a Parr Hydrogenator until there was no further drop in pressure. The reaction time was about twenty minutes. The catalyst was removed by filtration with suction and the filtrate was concentrated by distillation under reduced pressure in a bath of warm water. This concentrated solution was placed in an evaporating dish and allowed to stand until crystallization occurred. The crystalline mass was then dissolved in ether, filtered, and allowed to evaporate spontaneously. White crystals were obtained which were dried in a vacuum desiccator. The melting point of these crystals was 56–57°. A yield of 83% was obtained.

Anal.—Calcd. for $C_{11}H_{12}O_4$: C, 63.46; H, 5.77; Neut. Equiv., 208.2. Found: C, 63.2; H. 5.8; Neut. Equiv., 207.6.

Properties.—*β-(o-*Acetoxyphenyl)propionic acid is a white crystalline solid with a slight musty aromatic odor. One part dissolves in 100 parts of water at 20° It is very soluble in alcohol, ether, acetone, chloroform, and methanol at room temperature. It is insoluble in cold *n*-hexane and slightly soluble in hot *n*-hexane.

Its aqueous solution does not give a color reaction with ferric nitrate test solution.

Derivatives of β-(*o*-Acetoxyphenyl)propionic Acid. *β-(o-Hydroxyphenyl)propionic Acid.*—On acid hydrolysis a product was obtained that had a melting point of 82–84°. This melting point corresponds to that recorded for *β-(o-*hydroxyphenyl)propionic acid.

β-(*o*-Acetoxyphenyl)propionyl Chloride.—Five grams of *β-(o-*acetoxyphenyl)propionic acid were mixed with 5 Gm. of thionyl chloride and the mixture was gently refluxed for one hour. The excess thionyl chloride was driven off by distillation at 20 mm. pressure. The residue was then distilled at 1 mm. pressure and the material distilling over between 90 and 100° was collected. The compound gave a negative test for phenols with ferric nitrate test solution. The pure distillate had a boiling point of 95° at 1 mm. pressure. The yield was 89%.

Anal.—Calcd. for $C_{11}H_{11}ClO_3$: C, 58.3; H, 4.9. Found: C, 59.3, H, 5.0.

Methyl β-(o-Hydroxyphenyl)propionate.—Ten grams of the *β-(o-*acetoxyphenyl)propionyl chloride were dissolved in an excess of methanol, and the mixture was refluxed for one hour in a water bath. The excess methanol was then distilled off, and the residue was distilled at 1 mm. pressure. The material which distilled over between 120 and 130° was collected. On cooling, white crystals formed which had a melting point of 40–41°. The yield was 45%.

This substance gave a typical test for phenols with ferric nitrate T.S. The positive result indicates that the acetyl group is removed in this process.

Anal.—Calcd. for $C_{10}H_{12}O_3$: C, 66.7; H, 6.7. Found: C, 66.8; H, 6.8.

β-(o-Hydroxyphenyl)propionamide.—Five grams of *β-(o-*acetoxyphenyl)propionyl chloride were added slowly through a dropping funnel to 25 ml. of concentrated ammonia water in an ice bath. The reaction mixture was allowed to stand for thirty minutes. A precipitate formed that was removed by filtration and then washed with a small amount of cold water. The crystals were dried and treated with benzene, which gave an oily product. This oily product was dissolved in hot water, filtered, and shaken vigorously with ice. A crystalline product was obtained which, after drying had a melting point of 92–93°.

This substance gave a typical test for phenols with ferric nitrate test solution. Here again the acetyl group was removed in the process.

Anal.—Calcd. for $C_9H_{11}O_2N$: N, 8.6. Found 8.5.

Routine Screening Test for Toxicity.—Before an investigation of the analgesic properties of *β-(o-*acetoxyphenyl)propionic acid on rats was begun, this compound was submitted to a routine screening test on mice.

The compound was suspended in a bland oil and was administered orally by a stomach tube. The dosage level was started at 0.5 mg./Kg.

TABLE I.—TOXICITY OF *β-(o-*ACETOXYPHENYL)PROPIONIC ACID IN MICE, ADMINISTERED IN OIL SUSPENSION ORALLY BY STOMACH TUBE

Dose, mg./Kg. of Body Weight	Deaths	Survivals
0.5	0	1
5	0	1
50	0	1
500	0	1
5,000	1	0

These results shown in Table I gave a clue to the dosage levels that could be used in experimental animals in a more extensive investigation.

Analgesic Properties of β-(*o*-Acetoxyphenyl)propionic Acid.—It was felt that additional studies on the toxicity of this compound and its possible analgesic effects on rats should be determined before making studies of its effects in human subjects.

In this investigation Carworth-Wistar strain rats were used exclusively. The rats were kept under constant temperature of 72° F. ± 1°, fed on a Purina diet, deprived of food for twelve hours prior to control and medication tests, but provided with water at all times.

Two test methods were used: a modification of the D'Amour and Smith method and of the Hart method (2–4); and the silver nitrate method (5).

Modification of the D'Amour and Smith Method and of the Hart Method.—Acetylsalicylic acid, salicylamide, aminopyrine, and *β-(o-*acetoxyphenyl)propionic acid were run under the same control conditions and experimental protocols.

The Dolorimeter (with a time clock in the same circuit) was used to supply test stimulus. This instrument was checked prior to the actual experiments to insure maximal accuracy. Numerous screening tests were carried out to determine the best stimulus to employ in the experiments. It was found that 300 millicalories gave reproducible results in consecutive series of test animals.

Five rats were used in each test group. Control readings were obtained by taking the time response in seconds necessary to elicit a tail-flick in each animal when 300 millicalories of heat was applied to the end portion of the tail. The tail was blackened with charcoal prior to the taking of all readings. Responses were obtained uniformly in the control tests within a three to five second interval when the

animals were first used; but as they matured, some showed control readings slightly in excess of five seconds. At the beginning, all animals were within a weight range of 80 to 100 Gm.

A mean average of the control readings in seconds was obtained for each of the animals to be used in a given test. Three readings, at thirty minute intervals, were used in computing the average control time for the tail-flick response.

A mean average of the average control readings for the five animals was obtained. The standard error was computed for this mean average and was multiplied by two, thus giving a range with 19/20 confidence limits. This procedure was followed with each control group of rats, and it provided a basis for comparison with the results obtained when the same stimulus was applied after administration of the compounds under study.

If the mean average of the highest readings in seconds, obtained within a period of one hundred and five minutes after a test drug had been administered to a group of rats, exceeded the value representing the mean average of the average control readings plus two times the standard error, it was considered to be significant and therefore represented analgesia. Readings were taken at intervals of fifteen, forty-five, seventy-five, and one hundred and five minutes. The highest reading was taken as a criterion, since the rate of absorption varies from one animal to another. Preliminary screening tests indicated that a level of 500 mg./Kg., orally, of acetylsalicylic acid was necessary to achieve evidence of analgesia by the method outlined above. Therefore, that was the dosage level employed for each of the drugs tested.

A tuberculin syringe was used for measurement of the drug suspension.

Since most of the drugs used are only slightly soluble in water, each drug was suspended in a tragacanth suspension to insure accuracy of dosage. A 0.75% concentration of tragacanth was found to be satisfactory for these drugs.

A summary of the findings of the analgesic effects of these drugs in a dosage level of 500 mg./Kg., orally, in suspension with tragacanth, is shown in Table II.

These results by this method of testing indicate that the analgesic effects of β-(*o*-acetoxyphenyl)-propionic acid are slightly greater than those of acetylsalicylic acid and slightly less than those of salicylamide.

TABLE II.—RESULTS OF STUDIES BY A MODIFICATION OF THE D'AMOUR AND SMITH METHOD AND OF THE HART METHOD

	Mean Average Control Time in Seconds, ± Two Times the Standard Error
Acetylsalicylic acid	3.6 ± 0.4 (3.2–4.0)
Aminopyrine	3.7 ± 0.5 (3.2–4.2)
Salicylamide	4.9 ± 0.6 (4.3–5.5)
β-(*o*-Acetoxyphenyl)propionic acid	3.7 ± 0.5 (3.2–4.2)
	Mean Average of the Highest Readings in Seconds
Acetylsalicylic acid	4.1
Aminopyrine	5.4
Salicylamide	6.3
β-(*o*-Acetoxyphenyl)propionic acid	5.4

Silver Nitrate Test Method.—Silver nitrate, 0.2 ml. of a 1% solution, was injected into the joint area of the right ankle of 15 rats. Twenty hours later five of the rats were given, orally, 500 mg./Kg. of acetylsalicylic acid and another five were given the same dosage level of β-(*o*-acetoxyphenyl)propionic acid. The remaining five rats served as controls. All fifteen rats exhibited definite signs of inflammation and swelling of the right ankle joints. At intervals of one hour the right ankle of each rat was subjected to five pin pricks in various areas where inflammation and swelling were evident. A definite squeal was taken as an index of absence of analgesia. The total number of squeals for each animal was recorded; and a mean average, plus or minus two times the standard error, was calculated for each group of animals.

A summary of the findings of the analgesic effects of these two drugs in a dosage level of 500 mg./Kg., orally, in suspension with tragacanth is shown in Table III.

TABLE III.—RESULTS OF STUDIES BY THE SILVER NITRATE METHOD

	Mean Average Response ± Two Times the Standard Error
Control	17.8 ± 2.8 (15.0–20.6)
Acetylsalicylic acid	6.6 ± 2.8 (3.8– 9.4)
β-(*o*-Acetoxyphenyl) propionic acid	6.4 ± 4.6 (1.8–11.0)

The results obtained by this method of testing indicate that the analgesic effects of acetylsalicylic acid and of β-(*o*-acetoxyphenyl)propionic acid are about the same. Also, it was observed that both drugs reduced inflammation and swelling in the rats injected with silver nitrate.

Toxicity of β-(*o*-Acetoxyphenyl)propionic Acid.—Dosage levels of 1,000 and of 1,500 mg./Kg. of body weight, orally, in suspension with 0.75% tragacanth were used in additional studies of its analgesic properties by modification of the D'Amour and Smith method and of the Hart method. These dosage levels in rats produced no deaths. A summary is shown in Table IV.

TABLE IV.—TOXICITY OF β-(*o*-ACETOXYPHENYL) PROPIONIC ACID ON RATS, ADMINISTERED ORALLY

Dose, mg./Kg. Body Wt.	Deaths	Survivals
500	0	5
1,000	0	5
1,500	0	5

These results indicate that the LD_{50} of β-(*o*-acetoxyphenyl)propionic acid is above a dosage level of 1,500 mg./Kg., orally. This dosage level is higher than the LD_{50} for acetylsalicylic acid. On the strength of these findings it was felt that we could proceed with the investigation in human subjects if the dosage levels were kept within the range in which acetylsalicylic acid is used.

Metabolism of β-(*o*-Acetoxyphenyl)propionic Acid in Human Subjects.—Two healthy normal adult

volunteers were used in a preliminary investigation. Each subject ingested 1 Gm. of the drug per day for four days; this was followed by a rest period of two days; then each subject took 2 Gm. of drug a day for five days; this was followed by a rest period of three days; then each subject took 4 Gm. a day for four days. The amount of drug taken on any one day was divided into three equal doses and was taken within a twelve-hour period. In one case the subject took 4 Gm. in one dose. A slight irritation in the stomach was noted about fifteen minutes after the drug had been taken but this soon subsided without producing nausea or vomiting. The urine was collected for twenty-four hours prior to ingesting the drug and then twenty-four-hour urines were collected on each day in which the drug was taken.

Paper Chromatography Analysis.—Spots of urine were placed one inch from the bottom of strips of Whatman No. 1 filter paper. The strips were then air dried and placed in a glass tank containing *n*-butanol saturated with 3 *N* ammonia water. The solvent was allowed to travel up the paper for twenty hours. At the end of this time the paper strips were removed from the tank and allowed to dry in the air. The strips were then viewed under an ultraviolet lamp for fluorescent spots. After the presence of the fluorescent spots had been determined, the paper strips were sprayed separately with one of three different reagents. Each test was run in triplicate. In one, the strips were sprayed with ferric nitrate test solution, in a second with Millon's reagent, and in a third with Bell's phenol reagent (6). Bell's phenol reagent had to be followed with a spray of 20% sodium carbonate solution in order for a color to develop.

The colors that developed with these reagents and the R_f values of the spots that were obtained were first run on three known compounds that had been postulated as possible metabolites. β-(*o*-Acetoxyphenyl)propionic acid is not included in these studies since it did not respond to these tests. The results obtained with the known compounds are shown in Table V.

TABLE V.—R_f VALUES AND COLORS OBTAINED WITH THREE DIFFERENT REAGENTS ON THREE KNOWN COMPOUNDS

	Salicylic Acid	Salicyluric Acid	β-(*o*-Hydroxyphenyl)-propionic Acid
Fluorescence under ultraviolet light	Blue	Blue	None
Color with ferric nitrate	Purple	Purple	None
Color with Millon's reagent	Yellow	Yellow	Yellow
Color with Bell's reagent (Na_2CO_3)	Blue	Blue	Blue
R_f values	0.49	0.19	0.46

The urine of the subjects taking β-(*o*-acetoxyphenyl)propionic acid was then run chromatographically. One heavy fluorescent spot and two light ones that had traveled up the paper, and another spot that had traveled up the paper that did not fluoresce or give a color with the ferric nitrate test solution were detected after either Millon's reagent or Bell's reagent had been added.

The heavy fluorescent spot gave a slight purple color with the ferric nitrate test solution; a yellow color with Millon's reagent; a blue color with Bell's reagent; and it had an average R_f value of 0.19. This spot corresponds to that given by salicyluric acid.

One of the light fluorescent spots gave no colors with any of the reagents and had an average R_f value of 0.35. This spot does not correspond to any of those of known composition and has not yet been identified.

The other light fluorescent spot gave no visible color with either the ferric nitrate test solution or with Millon's reagent. It did give a light blue color with Bell's reagent and had an average R_f value of 0.51. The R_f value approaches that of salicylic acid, but it does not give the typical colors of salicylic acid with the reagents that were used. This compound has not yet been identified.

The spot that did not fluoresce under ultraviolet light and gave no color with the ferric nitrate test solution but gave a yellow color with Millon's reagent and a blue color with Bell's reagent had an average R_f value of 0.46. This corresponds to the spot obtained with β-(*o*-hydroxyphenyl)propionic acid.

Two metabolites of β-(*o*-acetoxyphenyl)propionic acid that are eliminated in the urine and which have been identified are salicyluric acid and β-(*o*-hydroxyphenyl) propionic acid. Two other substances also eliminated in the urine have not as yet been identified. No free salicylic acid was found in the urine.

Normal urine run by the same method did not give any of the substances which have been described.

No satisfactory method has been developed for the qualitative and quantitative estimation of β-(*o*-acetoxyphenyl)propionic acid. It has been found that it fluoresces at a wavelength of 410 mμ.

The Effect of β-(*o*-Acetoxyphenyl)propionic Acid on the Elimination of Uric Acid and of Glucuronides.—The determination of uric acid eliminated in twenty-four hours after ingesting 1 Gm , then 2 Gm., then 4 Gm. of drug were run by two methods: namely, a direct method in which a uricometer was used, and by the Uricase method of Buchanan, Block, and Christman (7). The uricometer method gave slightly higher readings but since it was much easier to run it was used to obtain the data shown in Table VI.

The urinary output of glucuronides in the same samples was determined by the method of Fishman and Green (8). The color produced in this assay was measured in a Coleman junior spectrophotometer, model 6B, and the reading obtained was compared to a standard curve previously prepared. The results of these findings are included in Table VI.

From the results obtained there is indiction of a slight increase in uric acid elimination. However, all amounts of uric acid elimination are within normal limits. In glucuronide elimination there is a definite increase; and while the amounts of glucuronide elimination are not in direct proportion to the amount of drug ingested, it appears that from 20 to 30% of the drug ingested is conjugated as the glucuronide.

TABLE VI.—THE EFFECT OF β-(*o*-ACETOXYPHENYL) PROPIONIC ACID ON THE ELIMINATION OF URIC ACID AND GLUCURONIDES

Date	Dose, Gm./day	Uric Acid, Gm./Day		Glucuronides, Gm./Day	
		Subject I	Subject II	Subject I	Subject II
7/8	0	0.39	0.43	0.596	0.866
7/9	1	0.38	0.54	0.765	0.990
7/10	1	0.45	0.61	0.828	1.024
7/11	1	0.60	0.60	0.810	1.224
7/12	1	0.57	0.59	0.804	1.485
7/13	0	..	..	..	..
7/14	0	..	..	..	..
7/15	2	0.65	0.54	1.177	1.855
7/16	2	0.59	0.65	1.017	1.672
7/17	2	0.78	0.47	1.298	2.291
7/18	2	0.35	0.42	0.949	1.730
7/19	2	0.48	0.73	0.952	1.670
7/20	0	..	..	..	..
7/21	0	..	..	..	..
7/22	0	0.39	0.48	0.612	1.197
7/23	4	0.46	0.66	1.170	2.527
7/24	4	0.75	0.54	1.027	1.837
7/25	4	0.62	0.55	1.042	1.853
7/26	4	0.48	0.60	1.164	1.891

The Effect of β-(*o*-Acetoxyphenyl)propionic Acid on the Elimination of Corticosteroids.—An increase in plasma corticosteroids and in the urinary excretion of reducing steroids in salicylate-treated rheumatic fever patients was reported by Cawenberge and co-workers (9, 10). However, no consistent change in 17-ketosteroid excretion was obtained by these investigators.

In a more recent paper Herndon, *et al.* (11), reported that "acetylsalicylic acid alone over a forty-eight-hour period increased the urinary excretion of neutral 17-ketosteroids and both free and conjugated corticosteroids."

The urine samples assayed for uric acid and glucuronides were assayed for urinary corticosteroids. The 17-hydroxycorticoids were assayed by a modification method of Reddy, Jenkins, and Thorn (12).

The 17-ketosteroids were assayed by the method of Holtorff, *et al.* (13), and by the method of Drekter *et al.* (14).

The results of these determinations are shown in Table VII.

The results of this investigation do not show any unsual picture in the elimination of cortiscosteroids in the urine. The results are within the normal range.

CLINICAL STUDY OF β-(*o*-ACETOXYPHENYL)PROPIONIC ACID[1]

This compound had previously been taken by healthy volunteers in high dosage levels over prolonged periods of time for evaluation of tolerance and possibly toxic side effects. It was then given to arthritic patients, in an attempt to evaluate its analgesic and possibly its antiphlogistic effects.

Plan.—The compound was given to six arthritic patients (5 rheumatoid and 1 osteoarthritic) requiring continuous salicylate therapy for control of joint pain. The patients received 30 mg./lb. of body weight, divided into four doses daily. This dosage was maintained for ten days; then for a period of three days the patients were given an indistinguishable placebo; then they were switched back to this compound β-(*o*-acetoxyphenyl)propionic acid for another three days; then they were placed back on an indistinguishable buffered salicylate for four days. Neither the patients nor the nursing personnel were aware of the changes that were made in medication.

At the end of the first ten days of medication with this compound, blood samples were drawn two and one-half and twelve hours after the last evening dose for measurement of blood levels.

Discussion.—Four of the six patients treated with this compound had some kind of gastric intolerance in the initial treatment. This complaint cleared up in three of these patients with whom the ingestion of the drug was continued. The other patient was discontinued because of nausea and vomiting. Another patient was discontinued because of severe headache. To what extent this compound is to blame for the nausea and vomiting or for the severe headache is difficult to evaluate since the first patient was also on a steroid withdrawal program and the second patient continued to have bouts of headaches for weeks after the cessation of this drug.

In this limited study, all patients receiving this compound reported adequate relief from joint pain. Since there is no objective method of testing the analgesic effects of drugs, our estimation of analgesia is based solely on the patients' statements as to pain, stiffness, and general well-being.

The tolerance of a three-day period on placebos without exacerbation of joint symptoms in all four remaining cases is not entirely clear. It may be due to prolonged action of the compound but this is mere speculation. Unlike salicylates, this compound does not react with iron salts so that it can be detected in the blood by the conventional methods employed for salicylate determination. Hence, it has been impossible to determine the concentration of this compound in the blood by the methods we employed.

The clinical course of the patients on this compound was followed closely. No changes in respiration, temperature, pulse rate, white blood cell count, or sedimentation rate were noted during the entire study period.

Conclusions.—β-(*o*-Acetoxyphenyl)propionic acid appears to have analgesic action. Clinical trials (double-blind) on a larger scale and over a longer period of time will be necessary to prove or disprove this assumption.

It produces a rather high incidence of mild gastrointestinal intolerance in the initial stages of treatment which seems to disappear, in most cases, on continued use.

No toxic side effects were noted in this small series of tests. The case studies of these patients plus those of others now being treated with this compound will be reported in another journal.

DISCUSSION

It was postulated that one of the end products of the metabolism of β-(*o*-acetoxyphenyl)propionic acid would be salicyluric acid. This assumption was based on the well known theory in biochemistry of

[1] This clinical investigation was made by Dr. Heinrich A. M. Tenckhoff under the supervision of Dr. Theodore B. Bayles, Director of Research at the Robert Breck Brigham Hospital, Boston, Mass.

TABLE VII.—EFFECT OF β-(*o*-ACETOXYPHENYL)PROPIONIC ACID ON CORTICOSTEROID ELIMINATION IN URINE[a]

Dose, Gm.	Subject I				Subject II			
	T. V.[b]	Cr.[c]	17-KS[d]	17-OH[e]	T. V.	Cr.	17-KS	17-OH
0	900	2,060	7.7	5.3	1,200	1,630	7.8	7.7
1	1,000	2,080	10.2	3.1	1,100	2,100	7.5	5.4
1	1,100	1,450	8.4	1.7	1,400	1,850	10.7	5.5
1	1,000	1,460	8.3	0.8	1,600	1,820	9.1	8.1
1	1,300	1,800	8.5	1.0	1,500	2,010	12.3	14.4
0								
0								
2	750	1,560	6.2	1.0	1,000	2,080	7.4	5.8
2	1,000	2,080	6.5	2.6	1,500	2,030	10.8	7.1
2	850	1,700	6.9	6.9	1,500	2,940	15.5	6.9
2	1,900	1,870	8.6	5.4	1,000	1,850	8.9	4.3
2	1,500	1,390	5.4	4.5	1,000	1,790	10.0	4.8
0								
0								
0	850	..	5.6	2.3	700	..	9.3	4.8
4	1,300	1,540	5.9	2.6	1,100	..	8.9	1.2
4	1,400	1,738	7.2	3.7	1,150	..	10.2	3.9
4	1,000	..	6.7	2.9	1,300	2,035	12.5	5.0
4	1,500	1,658	7.7	2.4	1,550	1,870	9.2	2.1

[a] These figures were confirmed by William J. Reddy in Dr. George W. Thorn's laboratory at the Peter Bent Brigham Hospital, Boston, Mass. [b] T. V., the total volume in twenty-four hours. [c] Cr., creatinine. [d] 17-KS, 17-ketosteroids. [e] 17-OH, 17-hydroxycorticoids.

β-oxidation of phenyl-substituted aliphatic acids. It was not anticipated, however, that if salicyluric acid were found in the urine, salicylates would not be found in the blood. These findings in no way disprove the validity of the β-oxidation theory of phenyl-substituted aliphatic acids, but it does seem to "pinpoint" the site of β-oxidation of β-(*o*-acetoxyphenyl) propionic acid and perhaps of those related to it. It would seem that the site of β-oxidation of this compound is in the kidney.

The analgesic effects of this compound in the limited study of six arthritic patients were good relief from joint pain in all cases. The tolerance of a three-day period on placebos in three of the four patients who completed the entire study suggests a prolonged analgesic effect. This observation has aroused considerable interest for continued study.

A satisfactory method for the determination of this compound in blood should be devised before a more intensive study and evaluation can be undertaken. A study of its concentration, accumulation, and elimination in blood and in urine is needed. A spectrophotofluorometric method of assay for this compound is under investigation.

SUMMARY

A homolog of acetylsalicylic acid, β-(*o*-acetoxyphenyl)propionic acid, has been synthesized. It is more soluble in water than is acetylsalicylic acid and does not react with ferric nitrate test solution to give a colored solution.

It has an LD_{50} of more than 1,500 mg./Kg. of body weight when it is administered orally to rats.

It analgesic effects appear to be equal to the analgesic effects of acetylsalicylic acid in the same dosage level.

In the initial stages of ingestion at a dosage level of 30 mg. per lb. of body weight in human subjects, it produces a mild epigastric discomfort that seems to subside after the third day of continuous medication. The use of buffers is suggested but has not as yet been investigated.

A study of the fate of this compound in human subjects indicates that four different substances are eliminated in the urine. Two of these, salicyluric acid and β-(*o*-hydroxyphenyl)propionic acid, have been identified.

Salicylates have not been found in the blood. The site of β-oxidation of β-(*o*-acetoxyphenyl)-propionic acid appears to be in the kidney.

The clinical course of patients on this compound in the higher dosage levels shows no changes in respiration, temperature, pulse rate, white blood cell count, or sedimentation rates after a fourteen-day study period.

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Urinary Excretion Kinetics for Evaluation of Drug Absorption II*

Constant, Rate-Limited Release of Tetracycline After Ingestion By Humans

By EINO NELSON

In *in vivo* absorption studies in humans of a composition of tetracycline and mucic acid, it was found that absorption was rate-limited by the *in vivo* dissolution rate of this composition when it was administered as thin pellets whose surface area could be assumed to have remained constant after ingestion. The rate-limiting *in vivo* dissolution rate was calculated to be about 2 mg. tetracycline as the hydrochloride per sq. cm. of composition per hour.

THIS PAPER presents and discusses the results of studies on tetracycline in which constant, rate-limiting release of drug was established in absorption from the gastrointestinal tract of humans. The studies were conducted with a composition of tetracycline and mucic acid (TMAC). The bases for interpretations on the excretion rate measurement used in evaluation were described in the first paper of this series (1).

EXPERIMENTAL

Conduction of Tests, Assay, and Preparations of Dosage Forms.—All aspects of these procedures were essentially the same as previously described (2). Drug was ingested by humans in apparent good health on fasting stomachs. In one series of tests, each dose of drug consisted of two thin pellets or disks about 0.95 cm. in diameter and 0.15 cm. thick contained in 000 hard gelatin capsules with sodium bicarbonate added to insure separation of the pellets in the stomach after ingestion. The dose of tetracycline was 200 ± 10 mg. in each test in terms of the equivalent of the hydrochloride salt. The compaction process used in preparation of the pellets insured their solution from exposed faces after ingestion rather than disintegration. The geometry of the pellets indicated that release of drug should be at a constant rate in dissolution medium of constant composition, since the surface area of their faces was much larger than their peripheral surface area. In another test series using the same subjects, TMAC equivalent to 125 mg. of the activity of tetracycline hydrochloride was taken in a raspberry-flavored aqueous suspension.

Collection times for urine specimens and pertinent data on subjects are indicated in the tabulated data that follow later in this report.

Tetracycline Mucic Acid Composition.—This material was prepared by mixing stoichiometric quantities of tetracycline hydrochloride and disodium mucate in aqueous solution, collecting and washing the resulting precipitate, and drying under vacuum at room temperature over sulfuric acid. Repeated microbiological assays on several batches of composition indicated that this material possessed an average of 87% of the activity of the tetracycline hydrochloride used in its preparation. The theoretical potency of a composition of two moles tetracycline to one mole of mucic acid is 88%. The composition possessed a water solubility at 25° of about 0.6 mg./ml. by microbiological assay and saturated solutions of the composition at 25° assumed a pH of 3.20 as measured on a Beckman model G pH meter, freshly standardized at pH 4.01. The nature of the interaction between tetracycline and mucic acid is the subject of continuing investigation (3).

RESULTS AND DISCUSSION

Excretion Data.—The cumulative amounts of tetracycline excreted to various times after ingestion of 200 mg. (HCl equivalent) of TMAC in the constant surface pellets is shown in Table I. The mean cumulative amounts of tetracycline excreted to various times after ingestion of 125 mg. (HCl equivalent) of the TMAC in suspension were 2.5, 8.0, 15.2, 20.8, 27.1, and 32.9 mg. tetracycline hydrochloride equivalent at one, two, three, four, six, and eight hours, respectively. The individual data will be reported in another paper (3).

Interpretation of Excretion Data.—When cumulative plots of amounts of tetracycline excreted were constructed from the data collected on individual test subjects, it was found that in two cases the slope of the plots assumed a constant value between two and three hours after drug ingestion. This is shown in Fig. 1 for subjects B and T. When the averaged data from the six subjects participating in this particular test were plotted, the same occurrence was noted as shown in the lower curve of Fig. 2. These results indicated that an equilibrium was established between tetracycline absorption, distribution, and excretion in accordance with considerations previously discussed concerning the kinetic principles involved (4). As previously mentioned, due to the design of the pellet dosage form, the surface area exposed to medium at absorption sites could be expected to remain relatively constant during dissolution leading to constant rate release and hence establishment of the equilibrium condition noted. The slopes of excretion curves were 3.8 and 3.4 mg./hr., respectively, for subjects B and T and 4.5 mg./hr. for all six subjects combined. These values, divided by the fraction of tetracycline in circulation that was

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TABLE I.—CUMULATIVE MG. TETRACYCLINE AND ML. URINE EXCRETED TO VARIOUS TIMES FROM TMAC IN PELLET FORM[a]

Subject[b]	mg. Excreted, Hr.						ml. Urine, Hr.					
	1	2	3	4	6	8	1	2	3	4	6	8
E (39–77)	2.7	9.2	16.9	23.0	33.5	42.8	110	340	500	590	780	970
S (33–57)	0.8	5.0	12.0	20.1	31.6	38.9	90	260	420	590	710	870
B (28–82)	0.4	2.7	5.8	9.3	16.6	23.9	40	80	120	160	250	350
T (23–75)	0.3	2.1	5.0	8.3	15.0	20.5	60	120	180	280	360	430
N (21–77)	0	0.4	1.1	2.2	4.8	8.0	310	350	380	410	480	550
H (26–73)	0.2	0.9	5.3	12.3	29.4	49.4	50	160	350	580	720	860
Mean	0.7	3.4	7.7	12.5	21.8	30.6	110	218	325	435	550	672

[a] Dose equivalent to 200 ± 10 mg. tetracycline hydrochloride. Excreted amounts are also in terms of hydrochloride equivalents.
[b] Quantities in parentheses are subjects' age in years followed by weight in Kg.

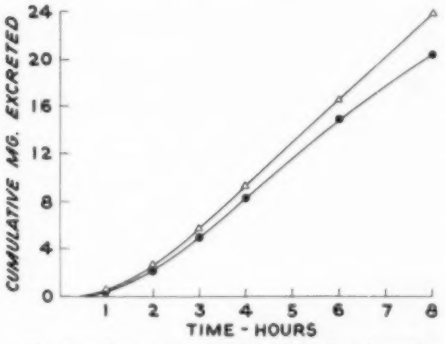


Fig. 1.—Constant excretion rate after ingestion of tetracycline mucic acid composition in constant surface pellets by subjects B and T. Solid circles, subject T; open triangles, subject B.

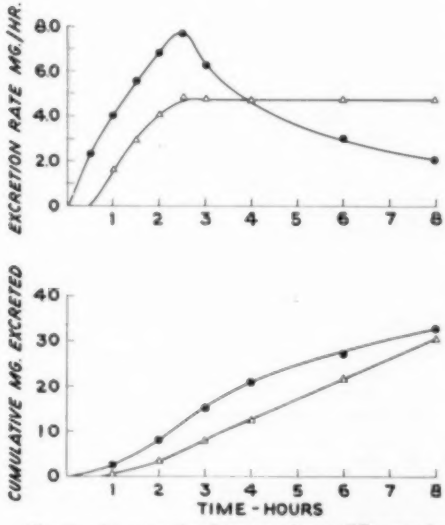


Fig. 2.—Lower pair of curves shows difference in cumulative amounts excreted when tetracycline mucic acid composition was given as a 125-mg. dose (HCl equivalent) in suspension, solid circles, and as constant surface pellets of this material in a 200-mg. dose (HCl equivalent), open triangles. Upper curves show excretion rates in the same cases.

excreted unchanged, gave the *in vivo* absorption rate (2).[1] In consideration of the conditions necessary for establishment of the equilibrium, these same rates must be equal to the *in vivo* dissolution rate of the pellets. Since the surface area of the pellet dosage was of the order of 3 sq. cm., the average *in vivo* solution rate was about 2 mg. tetracycline HCl/sq. cm./hr.

The constant rate release discussed persisted on the average for at least six hours after establishment, indicating that tetracycline, as TMAC, was available for dissolution and absorption in the gastrointestinal tract for at least the same period.

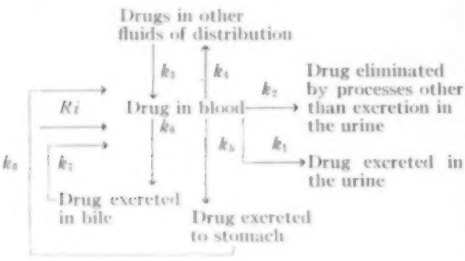
The upper curves of Fig. 2 which are plots of excretion rates indicate the nature of blood level curve that could be expected from the constant surface pellets of the TMAC under the conditions of this test. The corresponding cumulative amounts excreted and excretion rate *vs.* time curves for the 125-mg. dose (HCl equivalent) of the composition in suspension are included on both the lower and upper diagrams of Fig. 2 for comparative purposes. The higher excretion rates from the suspension preparation with a smaller dose of drug clearly indicate that *in vivo* solution rate was rate-limiting in absorption when the pellets were used and the correctness of the interpretation placed on the results.

The interpretation in this section concerning the estimated magnitude of *in vivo* dissolution and absorption rates from constant surface pellets are independent of the amounts of tetracycline that may have been involved in an excretion-absorption cycle via the bile. Tetracycline is found in the bile in high concentrations (5), but the volume of bile flowing is a small fraction of the volume of the other fluids of distribution and only small amounts would be expected to be involved in this cycle. The possibility also existed that tetracycline was involved in another excretion-absorption cycle via the stomach as a consequence of the pH-partition hypothesis (6). However, since an equilibrium between tetracycline absorption and excretion was apparently established, drug in these cycles was at equilibrium with drug in other fluids of distribution.

A generalized mathematical treatment to consider constant rate excretion from sustained release products presented by Campbell and co-workers (4) may be extended to include the bile and stomach cycles to show that this conclusion is correct. The absorption, distribution, and elimination of tetracycline may be portrayed by the following scheme when

[1] If this fraction was about 0.75, then multiplication of the excretion rates by 1.33 will give the absorption rates.

drug is released at a constant rate, Ri, at the absorption site:



In the scheme shown the k's with number subscripts are first-order rate constants for the indicated transfer processes and may be considered to incorporate partition factors when necessary, e. g., between drug in blood and drug in bile. The differential equations that describe distribution in the above scheme are:

$$dA_b/dt = Ri + k_3A_d - k_4A_b - k_2A_b - k_1A_b - k_5A_b - k_6A_b + k_7A_i + k_8A_e \quad \text{(Eq. 1)}$$

$$dA_d/dt = k_4A_b - k_3A_d \quad \text{(Eq. 2)}$$

$$dA_e/dt = k_5A_b - k_8A_e \quad \text{(Eq. 3)}$$

$$dA_i/dt = k_6A_b - k_7A_i \quad \text{(Eq. 4)}$$

$$dA_o/dt = k_1A_b \quad \text{(Eq. 5)}$$

In the above set of equations A_b, A_d, A_i, A_e, and A_o are, respectively, the amounts of drug in blood, other fluids of distribution, bile, stomach, and amount excreted. Since constant rate excretion was observed in the experiments, the amount of drug in blood A_b, must have remained at a constant value as a consequence of Eq. 5. With A_b constant, $dA_b/dt = 0$. Further, $dA_d/dt = 0$, $dA_e/dt = 0$, and $dA_i/dt = 0$. Since conditions other than this one would not allow $dA_b/dt = 0$, then

$$k_4A_b = k_3A_d \quad \text{(Eq. 6)}$$

$$k_5A_b = k_8A_e \quad \text{(Eq. 7)}$$

$$k_6A_b = k_7A_i \quad \text{(Eq. 8)}$$

and

$$0 = Ri - (k_1 + k_2)A_b$$

or

$$A_b = Ri/k_1 + k_2 \quad \text{(Eq. 9)}$$

Equation 5 may then be written as

$$dA_o/dt = (k_1/k_1 + k_2)Ri \quad \text{(Eq. 10)}$$

The coefficient of Ri on the right hand side of Eq. 10 has previously been noted to be equal to f, the fraction of drug excreted unchanged (4) so

$$\text{Constant excretion rate} = f\,Ri \quad \text{(Eq. 11)}$$

More complex distribution schemes than the above could be written. With constant excretion rate, Eq. 11 would still be obtainable from the equations describing the more complex scheme. For example, drug in blood could be considered to exchange between several more compartments without affecting the generality and conclusions obtained from the scheme discussed in detail here.

Absorbability of TMAC in In Vivo Solution Rate-Limited Trials.—The water solubility of TMAC was only about twice that possessed by tetracycline. In solution rate-limited absorption trials with the latter material with the same subjects and under the same conditions as used for TMAC trials, the mean maximum excretion rate observed was of the order of 1.8 mg./hr. followed by a steadily decreasing rate from about two hours after ingestion to the end of the tests (2). This contrasts to the constant rate of about 4.5 mg./hr. persisting for at least six hours after its establishment, two hours after ingestion of the same dose of TMAC. Since in both cases absorption was rate-limited by *in vivo* solution rate, the TMAC was capable of nearly constant rate dissolution after ingestion and the site of this dissolution was probably in the small intestine. The ability of solid TMAC to dissolve *in vivo* at a fair rate even though its water solubility was of a low order, was no doubt due to a physical-chemical property possessed by it but not by tetracycline.

Tetracycline possesses two acidic functions and one basic function (7). At hydrogen ion concentrations likely to be found in intestinal fluids, tetracycline would exist either as the zwitter ion, since the pH of its isoionic point is about 5.5,[2] or as the anion to a lesser degree, depending on the pH of the fluids. This means that the diffusion layer surrounding undissolved tetracycline would possess a pH near that of intestinal medium. No strong driving force for dissolution would exist under these conditions since the concentration in the diffusion layer would be a small value. On the other hand, the tetracycline mucic acid composition could be expected to possess an intrinsically strong driving force for dissolution in the intestinal fluids. The distinct acidic reaction possessed by the saturated solution of the composition as mentioned earlier in this report would necessitate a neutralization reaction to take place at the diffusion layer[3] of undissolved material. Since the hydrogen ion concentrations of intestinal and duodenal fluids are maintained by a bicarbonate buffer system, carbon dioxide would be evolved in this reaction. The gas evolution should hasten solution by facilitation of exchange of material between the diffusion layer and medium. This effect was observed *in vitro* by immersing one of the pellets used in the *in vivo* trials in a simulated intestinal fluid containing a small amount of sodium bicarbonate.

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[2] Based on the strongest acidic function.
[3] The thin layer of fluid that always surrounds a dissolving solid.

Studies on Local Anesthetics XXII*

The Basic Aroxy- and Aralkoxyacetanilides

By ALOIS BOROVANSKÝ, ALEŠ SEKERA†, and ČENĚK VRBA‡

The synthesis of nine isomeric phenoxy, benzyloxy, and phenylethoxy substituted diethylaminoacetanilides is reported. The activity in both surface and infiltration anesthesia and acute toxicity tests was determined.

CONTINUING OUR STUDY of the substituted acetanilides with anesthetic properties, we have prepared a new series of phenoxy, benzyloxy, and phenylethoxy derivatives of general formula I. Their structures are evident from Table II.

I, X = $-N(C_2H_5)_2$
II, X = $-Cl$
R = C_6H_5O, $C_6H_5CH_2O$, $C_6H_5CH_2CH_2O$

We have been led to this mode of substitution by observation of the interesting pharmacological properties of the diethylaminoethyl esters of aroxy- and aralkoxycarbanilic acids, the properties of which we have already reported (1).

The compounds were prepared according to the method previously described (2): the chloroacetanilides (II), obtained from the respective aryl amines, are converted to the corresponding basic aroxy- and aralkoxyacetanilides (I) by treatment with diethylamine.

EXPERIMENTAL[1]

Aromatic Amines.—These have been prepared by catalytic hydrogenation of the corresponding nitro compounds over Raney nickel according to the method described in a previous paper (1). The physical properties and the yields of the substances prepared correspond to the values there indicated.

Chloroacetanilides (II).—These have been prepared by the reaction of chloroacetyl chloride and the corresponding aryl amines in acetone in the presence of sodium carbonate (2). For the final reaction the crude products were used. The samples for the analysis were purified by crystallization from petroleum ether. The yields, the physical properties, and the analyses of the substances prepared are indicated in Table I.

Diethylaminoacetanilides (I; S250 – S258).—These have been prepared according to the method previously used (2) by the reaction of the corresponding chloroacetanilides with the diethylamine in anhydrous benzene. The hydrochlorides were precipitated from anhydrous ether solutions of bases by addition of ethereal hydrogen chloride. The yields, the physical properties, and the analyses of the substances prepared are included in Table II.

TABLE I.—SUBSTITUTED CHLOROACETANILIDES

No.	R	Yield, %	M. p., °C.	N, % Calcd.	N, % Found
1	2-C_6H_5O	97	77	5.35	5.43
2	3-C_6H_5O	67	107[a]	5.35	5.35
3	4-C_6H_5O	99	101	5.35	5.42
4	2-$C_6H_5CH_2O$	95	103	5.08	4.95
5	3-$C_6H_5CH_2O$	94	120	5.08	5.14
6	4-$C_6H_5CH_2O$	84	148[b]	5.08	5.12
7	2-$C_6H_5CH_2CH_2O$	96	59	4.83	4.60
8	3-$C_6H_5CH_2CH_2O$	95	63	4.83	4.99
9	4-$C_6H_5CH_2CH_2O$	76	124[c]	4.83	5.02

[a] From diluted ethanol.
[b] Purification of the sample for analysis: 100 cc. of the anhydrous 5% chloroform solution was filtered through a column of 10 Gm. of aluminum oxide (activity between I and II) and was then eluted by 300 cc. of the same solvent. The product obtained by evaporation of the eluate was crystallized from dilute ethanol.
[c] From ethanol.

PHARMACOLOGY[2]

The relative activity of the compounds in surface anesthesia (rabbit cornea, 0.01 *M* cocaine as standard) and infiltration anesthesia (intradermal application to guinea pigs, 0.02 *M* procaine as standard) was calculated from the molar concentration, experimentally found to give the same effect as the standard. The method has been described in detail by Vrba and Sekera (3, 4).

The toxicity was studied according to the method of Kärber (5) by determining the LD_{50} in white mice (strain H) by subcutaneous injection.

The results are presented in Table III.

DISCUSSION AND SUMMARY

Nine isomeric diethylaminoacetanilides, substituted in the benzene nucleus by a phenoxy, benzyloxy, and phenylethoxy group were pre-

* Received April 16, 1959, from the Department of Pharmaceutical Chemistry, Masaryk University, Brno, Czechoslovakia.
Paper XXI of this series: THIS JOURNAL, **48**, 402(1959).
† Inquiries regarding this article should be sent to A. Sekera. Present address: Laboratoire de Pharmacologie, Service de Chimie, 21 rue de l'École de Médecine, Paris VI^e, France.
‡ Department of Pharmacology, School of Veterinary Medicine, Brno, Czechoslovakia.
[1] All melting points were determined on a Kofler block and are corrected. Microanalyses were carried out by Mrs. Kleinová-Parolková.

[2] We are obliged to Miss M. Soukupová and A. Nováková-Niplová for technical assistance with the pharmacological tests.

TABLE II.—SUBSTITUTED DIETHYLAMINOACETANILIDES

No.	R	Base: Yield, %	Base: M. p., °C.	Hydrochloride: M. p., °C.	Hydrochloride: N, % Calcd.	Hydrochloride: N, % Found	Hydrochloride: Cl, % Calcd.	Hydrochloride: Cl, % Found
S 250	2-C_2H_5O	72	..	128	8.39	8.36	10.59	10.46
S 251	3-C_2H_5O	65	..	131.5[a]	8.39	8.30	10.59	10.39
S 252	4-C_2H_5O	56	..	123	8.39	8.53	10.59	10.61
S 253	2-$C_6H_5CH_2O$	61	..	125[b]	8.01	8.10	10.17	10.24
S 254	3-$C_6H_5CH_2O$	76	..	145	8.01	8.24	10.17	10.51
S 255	4-$C_6H_5CH_2O$	78	63[c]	123	8.01	7.85	10.17	9.94
S 256	2-$C_6H_5CH_2CH_2O$	79	..	147–149	7.72	7.97	9.77	9.79
S 257	3-$C_6H_5CH_2CH_2O$	82	..	150	7.72	7.55	9.77	9.62
S 258	4-$C_6H_5CH_2CH_2O$	81	59[d]	114	7.72	7.76	9.77	9.65

[a] Before the precipitation of the hydrochloride, the benzene solution of the anhydrous base (10.50) was filtered through a column of 15 Gm. of aluminum oxide (activity between I and II) and eluted with 300 cc. of the same solvent. The hydrochloride was crystallized from benzene-ether.
[b] From benzene-ether.
[c] *Anal.*—Calcd. for $C_{19}H_{25}O_2N_2$ (312.4): N, 8.97. Found: N, 8.93.
[d] *Anal.*—Calcd. for $C_{20}H_{28}O_2N_2$ (326.4): N, 8.58. Found: N, 8.67.

TABLE III.—PHARMACOLOGY OF SUBSTITUTED DIETHYLAMINOACETANILIDES

Substance	Relative Activity: Surface Anesthesia	Relative Activity: Infiltration Anesthesia	LD_{50} S. c. mg./Kg.
S 250	0.34	1.6	3,150
S 251	7.4	6.3	1,800
S 252	2.2	3.2	690
S 253	0.5	1.3	4,800
S 254	3.7	4.7	940
S 255	5.3	7.8	1,100
S 256	1	0.5	(6,000)
S 257	0.8	2	7,200
S 258	11	6.9	1,550
S 202 (lidocaine)	0.24	1.4	365
Cocaine	1	3.6	125
Procaine	0.15	1	630

pared and tested pharmacologically. The local anesthetic activities in surface and infiltration anesthesia and the acute toxicity were determined. It was found that most of the compounds are more active than cocaine in surface anesthesia and procaine in infiltration anesthesia. It was also found that they are, at the same time, up to ten times less toxic than these two standards.

From the results obtained, the following conclusions concerning the relations between the structure and the activity may be drawn:

1. If the activity of the substances prepared is compared with that of the model substance lidocaine[3] (S 202), it is to be noticed that lipophilization of the molecule by the substitution of the phenoxy, benzyloxy, and phenylethoxy group generally increases the activity. This increase is especially evident in surface anesthesia.

2. The substitution in the *ortho* position was seen in every case to be the least advantageous. Substitution of the phenoxy radical in the *meta* position produced the greatest increase in activity, whereas in the benzyloxy and phenylethoxy series the compounds substituted in the *para* position are most active.

3. The appreciably lower toxicity of these compounds in comparison with lidocaine (S 202) is surprising and significant. This reduction in toxicity affected by aroxylation and aralkoxylation as illustrated by the compounds described in this paper, is more pronounced than in a series of closely related basic carbamates reported previously (1). It is interesting to notice a certain parallel between the anesthetic activity and the toxicity; the least active substances are also usually the least toxic.

[3] The trade name for lidocaine is Xylocaine.

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Notes

A Note on the Application of Radioactive Isotopes to Studies of Drug Deterioration*

By ROBERT F. SCHIFFMAN† and JOHN E. CHRISTIAN

THE PROBLEM of determining the stability of a new drug or dosage form has long been a vexing one to the drug manufacturer. Great competition demands that the determination be carried out as quickly and as efficiently as possible. The general procedure followed at the present time is to place the drug under accelerated aging conditions and then to test for purity by ordinary chemical and analytical means. The results thus obtained are then applied to basic kinetic principles, in a manner similar to that outlined by Garrett and Carper (1). In this manner it is possible to predict shelf life.

The use of radioactive isotopes and tracer techniques suggests itself as a method of studying stability and shelf life since it offers great sensitivity and ease of detection, even in complex mixtures. In this work the application of this technique to the study of hydrolysis of C-14 carboxyl acetylsalicylic acid is shown.

EXPERIMENTAL

The Electrophoretic Separation of Salicylic Acid and Acetylsalicylic Acid.—C-14 carboxyl salicylamide was converted to C-14 carboxyl salicylic acid by alkaline hydrolysis using sodium hydroxide. The C-14 carboxyl acetylsalicylic acid was prepared using the method of Richter (2). This latter compound showed a specific activity of 0.1 μc./mg. All separations were carried out employing the Reco apparatus.[1] The separations were performed on Schleicher-Schuell 2045A filter paper strips. A buffer mixture composed of citrate-HCl, pH 4.0, ionic strength 0.40, was employed (3). Either 0.01 ml. or 0.02 ml. of the compounds to be separated were applied to the negative side of the strip. The strips were then run in an open strip manner, i. e., the strips were suspended on three plastic bridges so that no part of the strips touched the table of the electrophoresis apparatus and the ends hung free in the buffer. The entire apparatus was then enclosed with the plastic cover furnished with the apparatus, and made airtight by sealing the openings with Scotch electrical tape No. 33.[2] Electrophoresis was run for ten hours at 200 volts which produced a current of approximately 5 ma.

The spots were detected on the strips by either spraying with diazotized *p*-nitroaniline or by scanning with either the Actigraph apparatus[3] or the Forro apparatus.[4] The narrowest slit opening was used with each apparatus.

Positive identification of the curves of the radioactive materials was made by comparison to controls of unlabeled salicylic and acetylsalicylic acids which were run along with these strips. The spots on the control strips were located by spraying with diazotized *p*-nitroaniline.

It was found that acetylsalicylic acid and salicylic acid could be separated by means of electrophoresis. However, the method used here was found not to be quantitative, since large sample losses, as shown by a decrease in total radioactivity, were found.

The reason for the loss of sample in this procedure is unexplained, although a possible explanation is that the passage of current may cause some decarboxylation of the C-14 carboxyl salicylic acid.

Thus, the separations performed as a part of the kinetic study of aspirin described here are qualitative only.

Study of the Hydrolysis of C-14 Carboxyl Acetylsalicylic Acid.—One hundred milliliters of a solution of C-14 carboxyl acetylsalicylic acid were prepared in Sørensen citrate-sodium hydroxide buffer of pH 6.7 (4), such that it had a concentration of 6 mg./ml. One-half milliliter of solution was then placed in each of sixty 1-ml. rubber-capped vials.[5] Twenty vials were placed in each of three constant temperature baths, set at 25, 42 and 60°, ± 0.1°. The vials were removed from the baths, one at a time, at half-hour intervals. The last two vials in each bath were removed at intervals of approximately twenty-four hours. Upon removal the vials were immediately placed in ice water and stored in a refrigerator.

Twenty lambda of each sample was spotted on a strip and the strips were run, eight at a time, in the electrophoresis apparatus. The strips were then analyzed for radioactivity by scanning with the Forro radiochromatograph scanner. The graphs thus obtained were then examined for the breakdown of acetylsalicylic acid and the formation of the products of deterioration.

The results obtained after subjection of several of the C-14 carboxyl acetylsalicylic acid samples to 60° are shown in Fig. 1. These graphs represent both the distance of travel of the radioactive materials and the relative activity of the spots. The activities are related directly to the concentrations of the materials; thus it is possible to follow the course of breakdown of the acetylsalicylic acid and the subsequent formation of salicylic acid.

* Received August 21, 1959, from the Research Laboratories of the Bionucleonics Department, Purdue University.
† Present Address: DCA Food Industries, Inc., 45 West 36th St., New York, N. Y.
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[1] Model No. E-800-2, Research Equipment Corp., Oakland, Calif.

[2] Minnesota Mining and Manufacturing Co., St. Paul, Minn.

[3] Actigraph apparatus, model No. C-100, Nuclear Instrument and Chemical Corp., Chicago, Ill., 1.4 mg./cm.^{2}, mica window.

[4] Forro radiochromatograph scanner, model ACSHL, the Forro Scientific Co., Evanston, Ill., windowless, gas-flow counter.

[5] Wheaton injectable vials, No. 150-1, star line, with No. 1 sleeve stoppers.

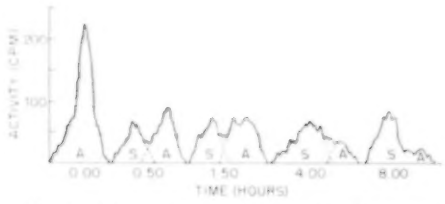

Fig. 1.—Electrophoretic patterns for the separation of C-14 carboxyl acetylsalicylic acid and C-14 carboxyl salicylic acid as obtained with the Forro radiochromatograph scanner, at 60°. A, acetylsalicylic acid; S, salicylic acid.

At 60° the breakdown is seen to occur rather rapidly. Only thirty minutes after zero time there appears a distinct curve for salicylic acid. The curves following this show a continual decrease in the amount of acetylsalicylic acid and an increase in the amount of salicylic acid.

The separation was not clear cut and resulted in some overlapping of spots. Throughout this work the *Rf*[6] value of acetylsalicylic acid was found to vary from 0.80 to 0.95 with the result that absolute definition of the spots often was very difficult. Such is the case here.

The curves obtained for the 42° and the 25° samples are not shown here as at both these temperatures very little hydrolysis was noticed over the first eight hours, and the curves, for the most part, resembled those for zero time at 60°. At 42° the appearance of a small peak for salicylic acid was first noticed at 2.50 hours. At 31.55 hours the sample was found to be almost entirely salicylic acid.

At 25° no change was noticed in the first nine hours. At 40.53 and 59.43 hours the sample had been converted entirely to salicylic acid, however.

As mentioned previously, the results obtained were qualitative only due to the difficulties encountered in the electrophoretic separations of the two compounds in question. However, despite the difficulties encountered in the separation procedure for the compounds studied here, the results obtained show that by radioisotope tracer techniques it is possible to follow the course of breakdown of medicinal agents, and the subsequent formation of the decomposition products. This fact is a significant one since it demonstrates the advantage of being able to study the breakdown of a drug or chemical without knowledge of the identity of breakdown products or the mechanism of degradation. The method also lends itself to the rapid determination of degradation products at an early stage in the decomposition. These factors combined with the ability to analyze for submicrogram quantities, even in complex mixtures, make this method of approach to the study of drug and chemical stability one which should have wide application in the future.

[6] The ratio between the distances traveled by a given compound and a reference substance contitutes the *Rf* value.

SUMMARY

A method of electrophoretic separation and identification was developed for the products of the thermal degradation of acetylsalicylic acid. The procedure, however, as used in this work was not quantitative. Experiments were performed to determine the progress of the thermally increased hydrolysis of acetylsalicylic acid by means of radioisotope tracer techniques. The use of radioisotopic tracers as a method of stability study was shown to have the advantages of being able to detect the most minute amounts of breakdown products, even in complex mixtures. It is also possible to determine the rate of breakdown without identifying the breakdown products or knowing the mechanism of degradation.

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Book Notices

Medicinal Chemistry. By WILBUR J. DORAN. Edited by F. F. Blicke and R. H. Cox. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1959. ix + 334 pp. 14.5 x 23 cm. Price $12.

In this fourth volume of a series of reviews prepared under the auspices of the Division of Medicinal Chemistry of the American Chemical Society a complete review of barbiturates, from their discovery to their present use as intravenous anesthetics, is competently presented. The review begins with the historical development of barbiturates followed by chapters dealing with the chemistry and pharmacology of these important medicinal chemicals. By the skillful use of 238 tables, an immense body of chemical data relating to all derivatives of barbituric acid is presented. The author and editors cite more than 1,200 references to the original literature. The value of the review is enhanced by a Formula Index to all compounds appearing in the Tables 1–230, with the exception of Tables 4–7 and 9–13. This Formula Index follows the same pattern as a *Chemical Abstracts* formula index.

Haemorrhagic Nephroso-Nephritis. By A. A. SMORODINTSEV, V. G. CHUDAKOV, and A. V. CHURILOV. Translated by Catherine Matthews. Pergamon Press Inc., 122 East 55th St., New York 22, N. Y., 1959. 124 pp. 14 x 21.5 cm. Price 60s. net.

This monograph is devoted to modern data concerning the etiology, epidemiology, clinical features, and pathological anatomy of haemorrhagic nephroso-nephritis by three authors whose systematic investigations qualified them as authorities on the disease.

Pathogenesis and Immunology of Tumours. Edited by G. V. VYGODCHIKOV. Translated by R. Crawford. Pergamon Press Inc., 122 East 55th St., New York 22, N. Y., 1959. vii + 258 pp. 14 x 21.5 cm. Price 80s. net.

This book contains a collection of papers by leading Russian specialists, and is sponsored by the Gamaleia Institute of Epidemiology and Microbiology. It has been produced to honor the 60th birthday of L. A. Zil'ber, and to commemorate his thirty-five years of service to science.

L. A. Zil'ber and his colleague have discovered facts of importance in their investigations on the etiology and immunology of tumors. They established the presence of specific antigens in neoplasms, and elaborated the original method for their demonstration, as well as a method for artificial immunization against experimental cancer with preparations not containing living cancer cells.

The collection has been made mainly from works by pupils and colleagues of L. A. Zil'ber. It includes, also, papers by other investigators working in the field of theoretical oncology. All these investigations deal with current problems in the pathogenesis of malignant tumors and their immunology.

Molecules and Mental Health. Edited by FREDERIC A. GIBBS. J. B. Lippincott Co., Medical Dept., East Washington Square, Philadephia 5, Pa., 1959. xii + 189 pp. 15 x 23 cm. Price $4.75.

Discussions of recent developments in the field of psychopharmacology possessing possible potential applications are presented. These papers, divided into two groups, discuss amines in relation to brain function and behavior and ACTH treatment of hypsarhythmia. The papers comprising this volume were those included in the programs of two 1958 conferences of the Brain Research Foundation.

International Textbook of Allergy. Edited by J. M. JAMAR. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, Ill., 1959. 639 pp. 16 x 24 cm. Price $17.50.

This medical textbook on allergy is a compilation of contributions by specialists under the following chapter headings: Anaphylaxis, Physiopathological role of histamine in man, Allergy and immunology, Definition-prevalence-predisposing and contributory factors, Diagnosis of allergic diseases, Provocation tests, Allergy of the upper respiratory tract—pollen, Fungi and miscellaneous inhalant allergens, Pathophysiology of bronchial asthma, Clinical aspects of bronchial asthma, Specific treatment of bronchial asthma, Nonspecific and symptomatic treatment of bronchial asthma, Status asthmaticus, Clinical use of corticotropin and adrenocortical steroids in allergic diseases, Use of antihistamines in allergic diseases, Psychosomatic aspects and psychotherapy in allergic diseases, Allergic diseases of the skin, Urticaria and angioneurotic oedema, Alimentary and gastro-intestinal allergy, Allergic diseases of the eye, Vascular allergy and collagen diseases, Allergy and hematology, Allergy of infancy and childhood, Migraine and allergic headaches, and Reactions to drugs.

Recent Progress in Hormone Research. Proceedings of Laurentian Hormone Conference 1958. Vol. XV. Edited by GREGORY PINCUS. Academic Press, 111 Fifth Ave., New York 3, N. Y., 1959. xiii + 504 pp. 15 x 23 cm. Price $12.50.

Papers presented at the 1958 meeting of the Laurentian Hormone Conference are included in this book. These papers are designed to reflect contemporary developments. A total of 14 papers with abstracts of discussions of each are grouped into those relating to the pituitary hormones, aspects of steroid chemistry and metabolism, aldosterone chemistry and physiology, and hormones and metabolism.

Synthetic Methods of Organic Chemistry. Vol. XIII. By W. THEILHEIMER. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1959. xvi + 600 pp. 15 x 23 cm. Price $27.50.

This thirteenth volume of a continuing series includes mainly work published between 1956 and 1958. Volumes XI–XIII are considered as the first three of the third series and an excellent index covering these three volumes is included. The selected material includes new methods, improvements of known methods, and also some old proved methods of synthesis of organic compounds. Volume XII of this series was noted in THIS JOURNAL, **47**, 897(1958)

Brimstone. The Stone That Burns. By WILLIAMS HAYNES. D. Van Nostrand Co., Inc., 257 Fourth Ave., New York 10, N. Y., 1959. xix + 308 pp. 14.5 x 21.5 cm. Price $5.95.

This book tells in narrative style the story of the American sulphur (sulfur) industry. It is based upon the 1942 publication "The Stone That Burns," and it is subtitled "The Story of the Frasch Sulphur Industry." Herman Frasch is referred to as a chemist and engineer who came to the U. S. when he was 19 years old and became laboratory assistant to Prof. John M. Maisch in the Philadelphia College of Pharmacy.

Chemistry of Carbon Compounds. Vol. IV. Heterocyclic Compounds. Edited by E. H. RODD. Elsevier Publishing Co., Amsterdam, 1959. Sole distributor for the United States, D. Van Nostrand Co., Inc., 120 Alexander St., Princeton, N. J. xviii + 656 pp. 14.5 x 22.5 cm. Price $24.

This volume contains the systematic survey of heterocyclic compounds starting with six-membered rings containing one oxygen or sulfur atom. A large variety of natural products are covered, including anthocyanins and flavone coloring matters, catechins and the brazilin group, rotenone, patulin, and the vitamin E group. Other chapters deal with coloring matters, both natural and synthetic, derived from heterocyclic systems previously described in Vol. IVA. Included in this group are the porphins, the chlorophylls, the phthalocyanines, and the cyanine dyes. One chapter is devoted to compounds containing less commonly occurring hetero atoms, such as silicon, phosphorus, and arsenic. The final chapter covers six-membered rings with two nitrogen atoms, and deals with the fundamental chemistry of the pyridazines, pyrimidines, and pyrazines.

Methods of Analytical Histology and Histo-Chemistry By EDWARD GURR. The Williams and Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1959. xv + 327 pp. 15 x 24 cm. Price $13.

This book describes in concise detail the methods used in the microscopical identification, by color reactions, of chemical groups and compounds as they occur in normal and pathological tissues. Procedures which require costly treatment such as X-ray or ultraviolet spectrography, freeze-drying, etc., have been omitted. The text is divided into ten main sections under the headings: Proteins and their component amino acid groups, Carbohydrates, Lipids, Nucleic acids, Pigments, Enzymes, Miscellaneous methods for organic substances, Inorganic substances, Sundry technical methods, Additional references, and an Appendix.

Methods of Biochemical Analysis. Vol 7. Edited by DAVID GLICK. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1959. ix + 353 pp. 15 x 23 cm. Price $9.50.

This volume, the seventh in the series, is the first one in which methods and techniques of biochemical analysis will be reviewed. The contributed chapters cover: Immunoelectrophoretic analysis, Analysis of basic nitrogenous compounds of toxicological importance, Spectrophotometry of translucent biological materials—opal glass transmission method, Determination of inositol, ethanolamine, and serine in lipides, Assay of lipoprotein lipase *in vivo* and *in vitro*, Determination of creatinine and related guanidinium compounds, Determination of ethyl alcohol in blood and tissues, and Determination of heparin. Subject and author indexes for volume 7 and a cumulative index for the series are appended.

Canadian Cancer Conference. Vol. III. Edited by R. W. BEGG. Academic Press Inc., 111 Fifth Ave., New York 3, N. Y., 1959. xiv + 461 pp. 15 x 23 cm. Price $12.

This volume of reports at the third Conference considers the recently gained knowledge of nucleic acid chemistry. Volume I explored: Experimental tumors, tumor-host relations, enzymes, and ionizing radiations; volume II covered: The cell, leukemia and chemotherapy, hormones and cancer, immunity, and basic mechanisms. The subjects in volume III are grouped under: Nucleic acids, genetics, viruses and tumors, and biology of cancer.

The Actinomycetes. Vol. I. Nature, Occurrence and Activities. By Selman A. Waksman. The Williams and Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1959. xi + 327 pp. 17 x 2.55 cm. Price $12.50.

This treatise is, in part, a summary of the investigations in soil microbiology during the past 45 years by Dr. Waksman and his associates. It is, without a doubt, an authoritative compilation of the important knowledge about the actinomycetes, which only about 20 years ago were dismissed as a "little-known group of microorganisms." A selected bibliography requiring 41 pages, an index to species of actinomycetes, and a general index are appended.

Fundamentals of Physical Chemistry. By H. D. CROCKFORD and SAMUEL B. KNIGHT. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1959. xvii + 463 pp. 14 x 21.5 cm. Price $6.95.

This book is based upon a 1950 edition entitled "Fundamentals of Physical Chemistry for Premedical Students," which was reviewed in THIS JOURNAL, **39,** 423 (1950). Prerequisites for this textbook are undergraduate general chemistry, qualitative and quantitative analysis, organic chemistry, and ability to solve quadratic equations and use logarithms. The revised text adds three chapters on thermodynamics, which includes a limited use of calculus. New topics include the change in the value of the equilibrium constant with temperature, the change of vapor pressure with temperature, and the energy of activation in reaction kinetics. The format and style of presentation is similar to that of earlier editions.

Progress in Drug Research. Vol. I. Edited by ERNST JUCKER. Birkhauser Verlag, Basel, Switzerland, 1959. 607 pp. 16.5 x 24 cm. Price sFr. 68.

This is the first volume of a planned series of annual reviews of current activities in fields of pharmaceutical research which include chemical, pharmacological and clinical aspects. Of the seven subjects covered in this volume, five are in German. The two English portions discuss Cholesterol and its relation to atherosclerosis (Lin and Chen of Eli Lilly) and Stereochemical factors in biological activity (A. H. Beckett, Chelsea College School of Pharmacy, London). The placebo problem is discussed in relation to clinical studies with many types of medication. The other subjects covered are: Die Ionenaustauscher und ihre Anwendung in der Pharmazie und Medizin; Die Chemotherapie der Wurmkrankheiten; Neuere Aspekte der chemischen Anthelminticaforschung; and Eine Übersicht der neuern Arzneimittle aus den letzten fünf Jahren.

The Chemistry of Heterocyclic Compounds. A Series of Monographs. Vol. XIII—*s-Triazines and Derivatives.* By EDWARD M. SMOLIN and LORENCE RAPOPORT. Interscience Publishers, 250 Fifth Ave., New York 1, N. Y., 1959. xxiv + 644 pp. 15 x 22.5 cm. Price $30 single, $28 subscription.

This is the thirteenth volume to appear of a series of monographs devoted to a comprehensive presentation of heterocyclic chemistry prepared by a group of authorities in this field. The subdivisions have been designed to cover heterocyclic compounds in their entirety by monographs which reflect the importance and interrelationships of the various compounds. This latest volume, in addition to an introductory chapter, covers Cyanuric acid and derivatives, Alkyl(Aryl)-*s*-triazines; Monohydroxy-, hydroxyamino-, dihydroxy-*s*-triazines and related compounds; Monoamino- and diamino-*s*-triazines; Ammelide, ammeline, and related compounds; Melamine and substituted melamines; Isocyanuric acid and derivatives; Condensed ring *s*-tirazine systems; Hexahydro-*s*-triazines; Hexamethylenetetramine; and *s*-Triazaborane and its derivatives.

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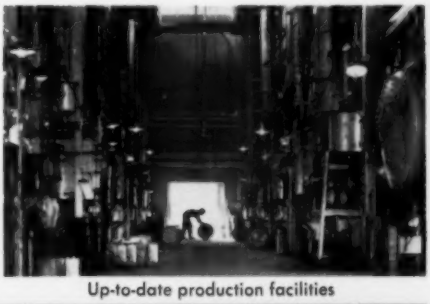

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